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ANALYSIS OF NEUROTRANSMITTERS, NEUROSTEROIDS AND THEIR METABOLITES IN BIOLOGICAL SAMPLES

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ACADEMIC DISSERTATION

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ABSTRACT

Neurotransmitters and neurosteroids are compounds that regulate the functions of the brain. The neurotransmitters dopamine (DA) and serotonin (5-HT) play a role in several psychological conditions, including schizophrenia, depression and anxiety. DA also has an important role in Parkinson's disease. Neurosteroids are involved in neurodegenerative diseases. In Alzheimer's disease and multiple sclerosis, the levels of neurosteroids are decreased in certain areas of the brain. Neurosteroids differ from classical neurotransmitters in that they are lipid-soluble and can easily cross the blood-brain barrier (BBB).

Neurotransmission can be studied in vivo by microdialysis, but as the concentrations of neurotransmitters in the microdialysates are very low, sensitive analytical methods are needed for their analysis. In this work an UPLC-MS/MS method was developed for the determination of 5-HT, DA, their phase I metabolites 5-HIAA, DOPAC and HVA, and their sulfonate and glucuronide conjugates. The method was validated and applied for analyzing human brain microdialysis and cerebrospinal fluid (CSF) samples. Intact glucuronide and sulfate conjugates were identified and quantified for the first time in the human brain.

The origin of the determined phase II metabolites in the brain is unknown. Even though sulfonate-conjugated compounds such as dopamine sulfonate (DA-S) and 5-HIAA-S were detected in the human brain, it is unclear whether they were locally formed or transported into the brain through the BBB from peripheral sources. The BBB permeation of DA-S was studied by administration of isotope ($^{13}C_6$)-labelled DA-S, which can be distinguished from endogenous DA-S by mass spectrometry, subcutaneously (s.c.) while brain microdialysis samples were collected and analyzed by UPLC-MS/MS. The fate of ^{13}DA-S in brain was followed by monitoring $^{13}C_6$-labelled DA-S metabolites and hydrolysis products. The results proved that DA-S permeates through the BBB, and indicated that DA-S finally either permeates through the BBB back to the peripheral circulation or is dissociated or metabolized by unknown mechanisms.

While the hydrophilic neurotransmitters DA and 5-HT are well suited for analysis by liquid chromatography coupled to atmospheric pressure ionization, the neurosteroids have more commonly been analyzed by methods based on gas chromatography (GC) coupled to ionization in vacuum. Recently GC has been combined to atmospheric pressure photoionization utilizing heated nebulizer microchips (μAPPI). We now constructed a simpler interface for combining GC to mass spectrometry (MS) using dopant-assisted

atmospheric pressure photoionization (APPI), utilizing commercially available hardware. The neurosteroids were analyzed as trimethylsilyl (TMS) derivatives, and the effect of different dopants (chlorobenzene, toluene and anisole) on the ionization and on the sensitivity of the method was investigated. Chlorobenzene was chosen as the best dopant, as the neurosteroid-TMS derivatives formed intense molecular ions with minimal fragmentation, while with toluene and anisole also protonated molecules were observed. The molecular ions of the steroids formed by APPI ionization showed fragmentation patterns in their MS/MS spectra similar to the patterns seen in corresponding spectra obtained by electron impact ionization (EI). Therefore the use of EI libraries could be possible, thus enabling the identification of a wide range of unknown compounds.

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I. Suominen, T, Uutela, P, Ketola, RA, Bergquist, J, Hillered, L, Finel, M, Zhang, H, Laakso, A and Kostiainen, R (2013). Determination of serotonin and dopamine metabolites in human brain microdialysis and cerebrospinal fluid samples by UPLC-MS/MS: discovery of intact glucuronide and sulfate conjugates. PLoS ONE 8(6): e68007

II. Suominen, T, Haapala, M, Takala, A, Ketola, RA, Kostiainen, R (2013). Neurosteroid analysis by gas chromatography – atmospheric pressure photoionization – tandem mass spectrometry. Anal. Chim. Acta 794, 76–81

III. Haapala, M, Suominen, T, Kostiainen, R (2013). Capillary Photoionization: A High Sensitivity Ionization Method for Mass Spectrometry. Anal. Chem. 85 (12), 5715–5719

IV. Suominen, T, Piepponen TP, Kostiainen, R (2015). Permeation of dopamine sulfate through the blood-brain barrier. Submitted to PLoS ONE 23.1.2015

The publications are referred to in the text by their roman numerals.

AUTHOR'S CONTRIBUTION TO THE PUBLICATIONS INCLUDED IN THIS THESIS

I. The experimental work was carried out by the author. The human brain microdialysis samples were provided by Jonas Bergquist and the human CSF samples by Jonas Bergquist and Aki Laakso. The UGT experiments were carried out by Hongbo Zhang and Moshe Finel with contribution from the author. The manuscript was written by the author with contributions from the co-authors.

II. The experimental work was carried out by the author with some contribution from Anna Takala and Markus Haapala. The manuscript was written by the author with contributions from the co-authors.

III. The experimental work was carried out by the author and Markus Haapala. The CPI interface was designed by Markus Haapala. The manuscript was written mainly by Markus Haapala with contribution from the author and co-authors.

IV. The experimental work was carried out by the author, except for the microdialysis experiments, which were carried out by the author, Petteri Piepponen and Marjo Vaha. The manuscript was written by the author with contributions from the co-authors.

ABBREVIATIONS

Ach	acetylcholine
A	aldosterone (11β,21-dihydroxy-pregn-4-ene-3,18,20-trione)
AP	allopregnanolone/ tetrahydroprogesterone (3α-hydroxy-5α-pregnen-20-one)
AN	androstenedione (androst-4-ene-3,17-dione)
API	atmospheric pressure ionization
APCI	atmospheric pressure chemical ionization
APPI	atmospheric pressure photoionization
μAPPI	miniaturized atmospheric pressure photoionization
Ch	choline
CI	chemical ionization
CNS	central nervous system
CORT	corticosterone (11β,21-dihydroxypregn-4-ene-3,20-dione)
CPI	capillary photoionization
CS	cortisone (17α,21-dihydroxypregn-4-ene-3,11,20-trione)
CSF	cerebrospinal fluid
DA	dopamine
DA-S	dopamine sulfonate
DA-3-S	dopamine-3-sulfate
DA-4-S	dopamine-4-sulfate
^{13}DA-3-S	$^{13}C_6$-dopamine-3-sulfate
^{13}DA-4-S	$^{13}C_6$-dopamine-4-sulfate
11-DC	11-deoxycortisol (17,21-dihydroxypregn-4-ene-3,20-dione)
DHEA	dehydroepiandrosterone (3β-hydroksiandrost-5-en-17-one)
5α-DHP	5α-dihydroprogesterone (5α-pregnane-3,20-dione)
DHT	dihydrotestosterone (17β-hydroksi-5α-androstan-3-one)
DTE	dithioerythritol
E	epinephrine, adrenaline
EC	electrochemical detection
EI	electron impact ionization
ESI	electrospray ionization
E2	β-estradiol (estra-1,3,5-triene-3,17β-diol)
E3	estriol (estra-1,3,5(10)-triene-3,16α,17β-triol)
E1	estrone (3-hydroxyestra-1,3,5(10)-trien-17-one)
FL	fluorescence
GABA	γ-aminobutyric acid
Glu	glutamate
HC	hydrocortisone/ cortisol (11β,17α,21-trihydroksipregn-4-ene-3,20-dione)
HILIC	hydrophilic interaction liquid chromatography
IE	ionization energy

IP	isopregnanolone (3β-hydroxy-5α-pregnen-20-one)
5-HT	serotonin
LLE	liquid-liquid extraction
LOD	limit of detection
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MSTFA	N-Methyl-N-(trimethylsilyl)trifluoroacetamide
MT	methyltestosterone (androst-4-en-3-one)
NE	norepinephrine (noradrenaline)
NH_4I	ammonium iodide
PA	proton affinity
PAPS	3'-phosphoadenosine 5'-phosphosulfate
PREG	pregnenolone (3β-hydroksipregn-5-en-20-one)
17-OH-PREG	17-hydroxypregnenolone (3β,17α-dihydroxypregn-5-en-20-one)
PROG	progesterone (pregn-4-en-3,20-dione)
RP	reversed-phase chromatography
s.c.	subcutaneous
SPE	solid-phase extraction
SIM	selected ion monitoring
SRM	selected reaction monitoring
SULT	sulfotransferase
T	testosterone (17β-hydroksiandrost-4-en-3-oni)
TMS	trimethylsilyl
5α-THDOC	5α-tetrahydrodeoxycorticosterone (3α,21-dihydroxy-5α-pregnan-20-one)
UDPGA	Uridine 5'–diphospho glucuronic acid
UGT	UDP-glucuronosyltransferase
UHPLC/UPLC	ultra-high pressure liquid chromatography
VUV	vacuum ultraviolet

1 INTRODUCTION

Neurons (nerve cells) form the building blocks of the central nervous system (CNS). Together, the billions of neurons in the brain communicate and process information through highly organized networks. The nerve impulse is transferred from a neuron to another with the help of neurotransmitters, which are endogenous chemicals that transmit signals from a neuron to a target cell across a synapse. Neurotransmitters are involved in maintaining the normal function of the brain, such as sleep and consciousness, as well as in some brain disorders, such as Parkinson's disease, epilepsy, schizophrenia, depression, anxiety, and dementia. The brain and the spinal cord together constitute the CNS, which lies inside the skull and the vertebral canal. A physical and biochemical barrier, the blood-brain barrier (BBB) formed by the capillary endothelial cells, prevents the access of many substances to the brain. Due to the BBB, the development of new drugs to target brain-related diseases proves challenging, since the BBB prevents entry into the brain of most drugs and endogenous compounds from the blood. Only small lipophilic compounds can diffuse passively through the BBB, while other compounds are usually only able to cross the BBB with the help of carrier proteins [1,2].

There are several groups of neurotransmitters with different chemical structures, including: monoamines (serotonin, 5-HT) and catecholamines (dopamine, DA and noradrenaline, NE), choline esters (acethylcholine, Ach), amino acids (glutamate, Glu and aspartate, Asp), γ-aminobutyric acid (GABA), peptides, and steroids [3]. As the neurotransmitters are involved both in the normal and pathological conditions of the brain, it is essentially important to develop methods by which they can be analyzed and quantitated in biological samples.

1.1 NEUROTRANSMITTERS AND NEUROSTEROIDS

Most neurotransmitters are synthezised from precursors in the axon terminals in the CNS, stored in vesicles and released to a synaptic cleft between the presynaptic and postsynaptic neurons. To terminate the signal, neurotransmitters are removed from the synaptic cleft by active uptake mechanisms or they are enzymatically broken down. DA and 5-HT are two of the main monoamine neurotransmitters in the brain. 5-HT is involved in the regulation of several physiological functions, including the sleep-wake cycles, body temperature, blood pressure, perception of pain, hormonal functions of the hypothalamus, and psychological functions, such as depression and anxiety [4,5], while DA has a role in Parkinson's disease, schizophrenia, depression, and the regulation of motoric movements [6,7].

Neurosteroids are generally classified as steroids with local function in the brain. They differ from other neurotransmitters in that they are lipid-soluble and can easily cross the BBB, and can therefore be formed in the brain *in situ* or in the periphery [3,8]. Peripherally formed neurosteroids are the corticosteroids, corticosterone (CORT) and aldosterone (A), as well as testosterone (T) and estradiol(E2); neurosteroids formed both in the periphery and the CNS are progesterone (PROG), allopregnenolone (AP), and pregnenolone (PREG), while the neurosteroid dehydroepiandrosterone (DHEA) found in the CNS is formed locally in the CNS. Steroids formed in the periphery with local function in the brain are sometimes called neuroactive steroids, and only those formed in the brain *in situ* neurosteroids, but in this thesis the term neurosteroid will be used for all steroids with local function in the CNS. The neurosteroids exert their effects by binding to the intracellular nuclear steroid receptors, but also by interaction with neurotransmitter-gated ion channels and membrane steroid receptors [9–11]. Neurosteroids regulate several cerebral functions, including protein synthesis, gene activation, and activity of the brain through the activation of gamma-aminobutyric acid (GABA) and N-methyl-D-aspartate (NMDA) receptors, as well as nicotinic, muscarinic and serotonergic receptors [11,12]. Neurodegenerative diseases have been shown to alter neurosteroid levels. In Alzheimer's disease and multiple sclerosis, for example, the levels of neurosteroids are decreased in certain areas of the brain [13].

1.1.1 PHASE I METABOLISM

5-HT is synthesized from the amino acid tryptophan, while the synthesis of DA starts from tyrosine. Both DA and 5-HT are metabolized by monoamine oxidase (MAO) to the phase I metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and 5-hydroxyindoleacetic acid (5-HIAA), respectively. DOPAC is further metabolized to homovanillic acid (HVA) by catechol-O-methyltrasferase (COMT) (Figure 1). Both DA and 5-HT, and their respective metabolites, can undergo conjugation with glucuronic acid or sulfonate mediated by catalysis with UDP-glucuronosyltransferases (UGTs) and sulfotransferases (SULTs), respectively.

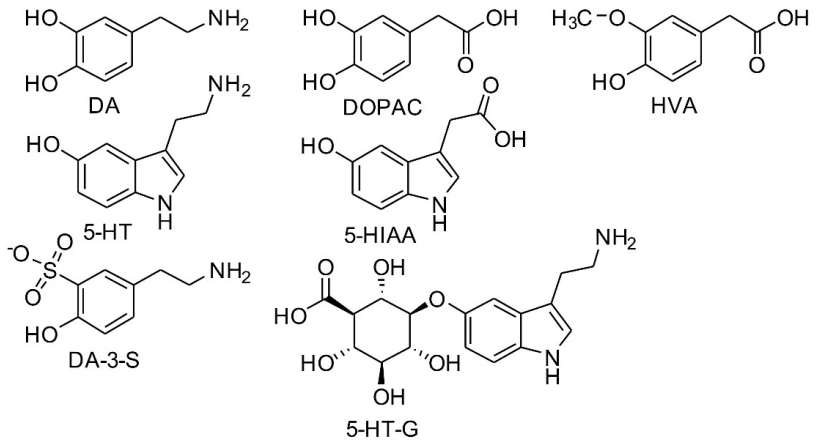

Figure 1 Structures of the studied neurotransmitters and their phase I metabolites, as well as the sulfate conjugate of DA and glucuronide conjugate of 5-HT-G.

All steroids are biosynthesized from cholesterol; the neurosteroids are biosynthesized from cholesterol or from steroidal precursors imported from peripheral sources [8,14]. The rate-limiting step in the synthesis of steroid hormones is the conversion of cholesterol to pregnenolone. The neurosteroids are mainly metabolised by cytochrome P450 oxidase enzymes, such as CYP3A4, to other steroids (Figure 2). Several enzymes involved in the metabolism of steroids, such as 5α-reductase, 3α-hydroxysteroid dehydrogenase and 17β-hydroxysteroid dehydrogenase, are present in the human brain [15].

1.1.2 PHASE II METABOLISM: CONJUGATION WITH GLUCURONIC ACID OR SULFONATE

The UGTs are a family of enzymes that catalyze the glucuronidation of various compounds, and have an important role in the detoxification of a large number of xenobiotic and endogenous compounds [16,17]. Many UGTs are expressed mainly in the liver, but UGTs are also expressed in other organs, and some of the UGTs are expressed only, or mainly, in extra-hepatic tissues, such as the gastrointestinal tract, the olfactory mucosa, adipose tissue, and the kidneys. Small amounts of UGT mRNA have also been found in several other tissues, including the heart, adrenal gland, trachea and brain [17–19]. The UGTs catalyze the transfer of glucuronic acid from UDPGA to various compounds, in order to make the compounds more hydrophilic and thus easier to eliminate from the body.

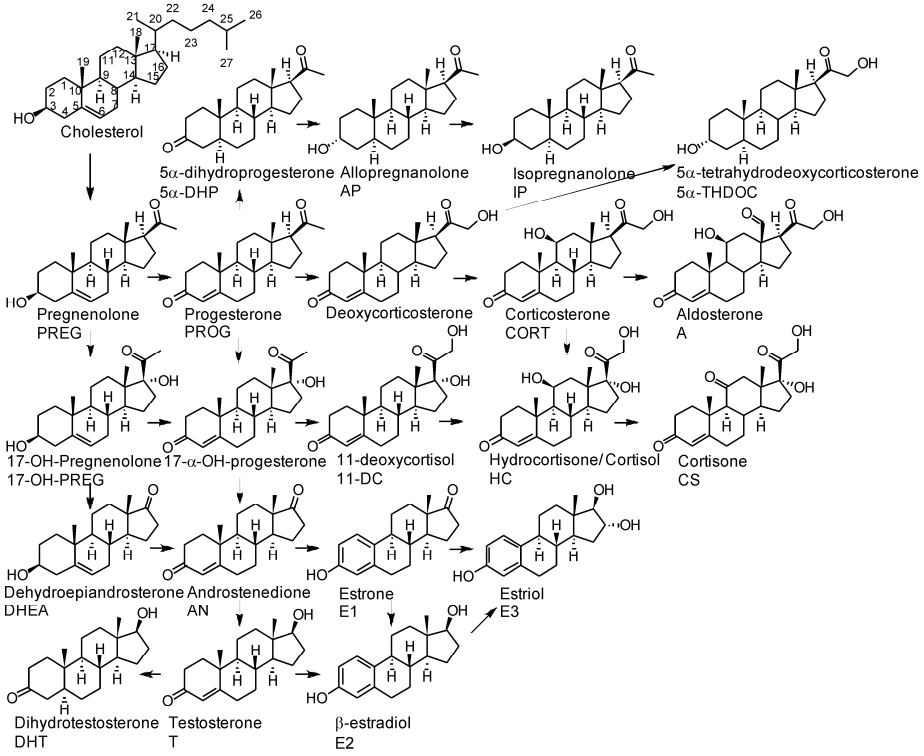

Figure 2 Biotransformation routes for selected steroids. Modified from [20].

Another family of enzymes, the SULTs, catalyze the sulfonation of different compounds, and are equally important in the metabolic conjugation of xenobiotics and endogenous compounds. There are 13 different human sulfotransferases that can be divided into three families, SULT1, SULT2, and SULT4. Similar to that of the UGTs, the expression of SULTs is tissue-specific. The liver is the most abundant site for most SULTs expression, although the intestine, lung, kidney, and brain also express SULTs [21,22]. The SULTs catalyse the transfer of a sulfonate group from 3'-phosphoadenosine-5'-phosphosulfate (PAPS) to various compounds, mainly attaching the sulfonate group to hydroxyl or primary amine groups [23].

Intact glucuronide conjugates of DA and 5-HT, and sulfonate conjugates of their phase I metabolites DOPAC, HVA, and 5-HIAA have been found in rat brain [24], and intact sulfates of 5-HIAA and DA in human brain [25]. The glucuronide and sulfate conjugates have been considered inactive, but recently the activity of glucuronide and sulfate conjugates of a few substances have been shown. The glucuronide or sulfate conjugates of DHEA, PREG [26,27], and morphine [28,29] are thought to be more active than the parent compounds in modulating their pharmacological effects in the CNS. The neurosteroid dehydroepiandrosterone (DHEA) also has an active conjugated metabolite,

DHEA sulfate, which is thought to be involved in several physiological and neuroprotective processes as well as in the regulation of the excretion of catecholamines [26]. The glucuronidation of steroids has been suggested to have neuroprotective effects by eliminating high steroid concentrations that have been linked, for instance, to breast cancer [30], and DA sulfonation has been suggested to serve as a transport form of DA into cells, where free DA could be regenerated [31].

1.2 ANALYSIS OF NEUROTRANSMITTERS

The concentration of neurotransmitters can be sampled directly in vivo from different parts of the brain using different techniques; microdialysis is the most common. Neurotransmission can also be studied in brain tissue, which enables the study of different regions of the brain, but has to be performed *post mortem* in most cases. In addition, cerebrospinal fluid (CSF) can also be analyzed, but CSF concentrations reflect average concentrations accumulated from all brain regions, and analysis of a specific brain area is not possible. In animal studies, often in rats, it is possible to select the appropriate matrix based on the researcher's needs. In humans, however, the use of ventricular CSF, brain tissue, or microdialysis of the extracellular fluid are possible only during certain neurosurgical operations or *post mortem*. Lumbar puncture is the most important and widely used diagnostic tool in the study of monoamine metabolite concentrations in humans [32].

1.2.1 BRAIN EXTRACELLULAR FLUID

When neurotransmitters are released, a small fraction leaks out of the synaptic clefts to the extracellular matrix. Thus, the concentration of a neurotransmitter in the extracellular fluid is a relatively reliable measure of a particular neuronal activity [33]. The concentration of neurotransmitters in brain extracellular fluid have been measured by different techniques.

Prior to the use of microdialysis, other in vivo techniques were employed, such as the cortical cup, which is a cylinder that is placed above a small hole in the studied animal's head, and enables sampling of chemicals released on the surface of the cortex. The extracellular levels of AK, GABA, and Glu have been determined by the cortical cup method [34–36], but the drawback to this method is that it is limited to only a certain region of the brain.

In voltammetry, an electrode is placed in the tissue examined and the current caused by oxidation of analytes is proportional to their concentration. Fast-scan cyclic voltammetry makes measurements of changes in the extracellular level of dopamine or other monoamines at millisecond time resolution possible. The narrow probes (diameter 5–20 μm) cause minimal tissue damage, but the drawbacks are relatively poor sensitivity and selectivity. Voltammetric measurements are usually in the μM or high nM concentrations

range, and thus do not allow assessment of basal levels [37]. The technique is limited to analytes that are electroactive and can be oxidized, such as monoamines. Fast-scan cyclic voltammetry is a differential technique and thus only changes in analyte concentration can be measured; the technique is not suited for measuring long-term changes or constant basal concentrations [38]. Another drawback to voltammetry is that no drugs or other substances can be locally applied in the tissue examined [39].

Push-pull perfusion can be considered to be a precursor to the microdialysis technique. It includes two cannulas implanted in a certain brain region, using an open flow system. Various substances can be sampled and detected by this technique, and it has been used for sampling of neurotransmitters and other endogenous substances. The push–pull perfusion method is an open system where the perfusion fluid directly contacts the tissue under study at the tip. In earlier designs the size of the probe and the flow at the tip often caused tissue damage or bacterial contamination at the site of perfusion [40]. However, to overcome this problem, miniaturized push–pull cannulae have been constructed utilizing low nl/min flowrates for successful analysis of Ach, 5-HT, DA, GABA, Glu, and Asp [41,42].

In microdialysis a cannula with a tip covered with a semipermeable membrane is used. An advantage compared to the push-pull technique is that the perfusion fluid (artificial CSF) does not come directly into contact with the extracellular fluid. The dialysis membrane is permeable to small molecules but not to macromolecules such as proteins. Endogenous compounds are sampled via the probe, because the levels of neurotransmitters and metabolites are higher in the extracellular space than in the perfusion fluid [43,44]. A drawback of microdialysis is the fact that the recovery of the measured substances in the microdialysis probe can be low and differ according to the dialyzed molecule. Due to the low flow rates used, time resolution is low, samples are usually collected in a time frame of 20-30 minutes [39,40]. The small sample volumes and the fact that the concentrations of most neurotransmitters are in the pM or low nM range leads to a demand for highly sensitive analysis techniques, such as LC-MS/MS.

Microdialysis has been largely used in the analysis of neurotransmitters and their metabolites in the central nervous system of laboratory animals, mainly in rats and mice [24,45–52]. In the human brain microdialysis has been used to monitor neurointensive care patients with subarachnoid hemorrhage, traumatic brain injury, thromboembolic stroke, or epilepsy [53–56]. Microdialysis of the human brain has also been performed in order to sample extracellular dopamine in the human amygdala during the performance of cognitive tasks in patients undergoing evaluation for epilepsy surgery [57], during thalamotomy intended to relieve tremor in patients with Parkinson's disease [58], in patients undergoing deep brain stimulation surgery for advanced Parkinson's disease [59], in patients with pharmacologically intractable seizures that underwent implantation of intracranial depth electrodes [60], and in patients with severe head injuries or subarachnoid

haemorrhage, in which microdialysis probes were placed together with a ventriculostomy catheter for drainage of CSF [61].

1.2.2 CEREBROSPINAL FLUID

In addition to the brain extracellular fluid, neurotransmitters have commonly been analysed in the CSF, especially in humans [53,62–67]. CSF is produced mainly from arterial blood by the choroid plexuses of the lateral and fourth ventricles of the brain. Around 80% of the CSF is secreted by the choroid plexuses, with the remaining 20% coming from the interstitial fluid of the brain. The epithelial cells in the choroid plexuses, and the tight junctions between them, form the blood-CSF barrier. At the blood-CSF barrier, some transporter proteins similar to those at the BBB have been identified [1,68–70].

The monoamine metabolite concentrations in the CSF reflect average concentrations accumulated from all brain regions together with the regional changes that occur within the spinal cord. Some neurotransmitters, such as 5-HIAA and HVA, have a rostrocaudal gradient, i.e. the concentrations differ depending on the site of sampling and changes if multiple samples are drawn from the lumbar section [32,71,72]. The CSF contains several proteins that can also be found in plasma, such as albumin, although at smaller concentrations [69,73].

1.2.3 SAMPLE PRETREATMENT

Neurotransmitters such as serotonin, dopamine, and their metabolites are small hydrophilic compounds, and have thus been analysed mostly by analysis techniques based on liquid chromatography with electrochemical or mass spectrometric detection.

Microdialysis samples require virtually no pretreatment, but if MS detection is used, the inorganic salts of the artificial CSF used as perfusion fluid have to be prevented from entering the mass spectrometer, as they might cause ion suppression [43]. As CSF samples contain proteins, they have to be removed prior to analysis by liquid chromatography, usually by protein precipitation and centrifugation [32,67,74] or by ultrafiltration [25,75–77]. Concerning brain tissue samples, the matrix is much more complicated and contains, for example, several different lipids, that might clog the analytical HPLC column and suppress ionization in mass spectrometry (ESI) [55,78]. Neurotransmitters are often extracted from brain tissue by homogenization of the sample and denaturing of proteins followed by analysis of the supernatant, usually after centrifugation and/or filtration [79–82], liquid-liquid extraction (LLE) [83], or solid-phase extraction (SPE) [84,85].

1.2.4 LIQUID CHROMATOGRAPHY – MASS SPECTROMETRY

Liquid chromatography using reversed-phase (RP) columns, most commonly C-18-columns, have previously been widely used [45,49,50,80–83,85–89]. Since the monoamines are polar molecules with low molecular weights, they are poorly retained in reversed chromatography. Ion-pair agents such as sodium octyl sulfate are generally employed in the mobile phase in order to increase retention times of polar analytes such as DA and 5-HT. However, these additives are not volatile, and therefore not compatible with MS detection. The phase II metabolites of the neurotransmitters are even more hydrophilic than the unconjugated parent compounds, and therefore hydrophilic-interation (HILIC) [42] or pentafluorophenyl-propyl columns have been utilized more recently in the analysis of intact phase II metabolites [24,25,47,48].

Mass spectrometric (MS) methods have become more common in neurotransmitter analysis, mostly coupled to high-pressure liquid chromatography (HPLC) or ultra-high pressure chromatography (UHPLC), utilizing atmospheric pressure ionization(API), usually electrospray (ESI) which is most suitable for polar compounds (Table 1). Since neurotransmitters are present in the brain at very low levels, usually triple quadrupoles in selective reaction monitoring (SRM) mode have been used, either in positive or negative mode [24,25,42,47,83,87]. Analysis of neurotransmitters by MS usually does not require derivatization, but derivatisation with benzoyl chloride [45] or deuterated acetaldehyde [90] has been used prior to analysis by LC-MS/MS to improve sensitivity and selectivity. A major advantage of MS using ESI is that the analysis of intact phase II conjugates (glucuronide and sulfonate conjugates) of neurotransmitters is possible [24,25,47].

1.2.5 OTHER DETECTORS

In addition to mass spectrometry, neurotransmitters are often analysed using other detectors, such as ultraviolet (UV), electrochemical (EC), and fluorimetric (FL) detection. As monoamine neurotransmitters are easily oxidized, electrochemical (EC) detection has been widely used (Table 1) [46,49–51,80,81,89]. EC detection is a form of voltammetry, and is based on the oxidation (or reduction) of the analytes in the mobile phase. EC is generally more sensitive than UV detection, and has thus been more widely used in neurotransmitter analysis.

Also fluorometric detection is generally more sensitive than UV detection, but often requires derivatization of the analyte with a fluorophore-containing reagent. For DA fluorometric detection utilizing derivatisation with diphenylethylenediamine has been proved more sensitive than UV detection [86], and also other monoamine neurotransmitters have been analysed with fluorescence detection, usually after derivatization [62,63,88,91] or utilizing their native fluorescence [82].

Table 1. *Examples of studies in which neurotransmitters have been analyzed in human and rodent brain*

Analytes	Method	Column	Ionization	Matrix	Sample	Ref.
DOPAC, 5-HIAA, 5-HT, NE, Glu	HPLC-EC	RP(C-18)		Human brain (temporal lobe cortex)	MD	[92]
DA, GABA, Glu	HPLC-EC	RP(C-18)		Human brain (subthalamic nucleus)	MD	[59]
DA, DOPAC, HVA, 5-HT, 5-HIAA + sulf, gluk	UPLC-MS/MS	PFP	ESI +/-	Human brain (prefrontal cortex), CSF (lumbar and ventricular)	MD, CSF	Publication I
DA, DOPAC, HVA, 5-HT, 5-HIAA, NE, 3-MT + others	HPLC-EC	RP(C-18)		Human brain, 16 areas CSF (lumbar and ventricular)	H, CSF	[93]
DA, DOPAC, HVA, 5-HT, 5-HIAA, NE, E	HPLC-EC	RP(C-18)		Human brain, 21 areas	H	[94]
HVA, DOPAC, 5-HIAA, 5-HT, MHPG	HPLC-EC	RP(C-18)		Human CSF (lumbar)	CSF	[65]
DA, DA-S, HVA, DOPAC	HPLC-EC	RP(C-18)		Human CSF (ventricular)	CSF	[95]
DA, DOPAC, HVA, 5-HT, 5-HIAA, NE, 3-MT, GABA, Glu + other amino acids	NanoLC-MS/MS	RP(C-18)	ESI +	Rat brain (ventral tegmental area, nucleus accumbens)	MD	[45]
DA, DOPAC, HVA, DA-S, DA-G, E, NE	HPLC-MS/MS	PFP	ESI +/-	Rat/mouse brain (striatum, nucleus accumbens)	MD	[47]
DA, DOPAC, HVA, 5-HT, 5-HIAA + sulf, gluk	HPLC-MS/MS	PFP	ESI +/-	Rat brain (striatum)	MD	[24]
DA, 5-HT, Ach, adenosine	LC-MS/MS	PFP	ESI +	Rat brain (nucleus accumbens)	MD	[48]
DA, 5-HT, Ach, GABA, Glu, Asp	CapLC-MS/MS	HILIC	ESI +	Primate cerebral cortex	Push-pull extract	[42]
DA, DOPAC, HVA, 5-HT, 5-HIAA, Ach, Ch, Glu, GABA	UHPLC-MS/MS	RP(C-18)	ESI +	Rat brain tissue, 4 areas	H	[87]
DA, DOPAC, HVA, 5-HT, 5-HIAA, NE, MHPG, MHPG-S	HPLC-MS/MS	RP(C-18)	ESI +/-	Rat brain tissue (whole brain)	H	[83]
DA,DOPAC,HVA,5HT,5HIAA,3-MT	HPLC-MS/MS	RP(C-18)	ESI +/-	Rat brain tissue, 5 areas	H	[85]

MD=microdialysate, H=homogenate, Glu=glutamate, Gaba=γ-aminobutyric acid, Asp=aspartatate, CapLC=capillary liquid chromatography, E=epinephrine, NE=norepinephrine, MHPG=4-hydroxy-3-methoxyphenylglycol, MHPG-S=MHPG-sulfonate, 3MT=3-methoxytyramine, PFP=pentafluorophenylpropyl, RP=reversed-phase

1.3 ANALYSIS OF NEUROSTEROIDS

In contrast to the hydrophilic neurotransmitters, neurosteroids are lipophilic compounds and have traditionally been analysed mostly by gas chromatography – mass spectrometry (GC-MS). However, LC-MS techniques have also become more common in neurosteroid analysis as they allow the analysis of intact steroid conjugates. Additionally, steroids can be analysed by UV detection, and also fluorescence detection after derivatisation has been performed [96].

1.3.1 MATRIXES

As steroids are lipophilic compounds, microdialysis is not suitable for sampling of neurosteroids, and therefore they are often analysed in CSF [97–101]. The levels of neurosteroids in CSF have been shown to correlate with levels in the brain [98]. Due to their lipophilic properties, neurosteroids can permeate the BBB, and therefore analysis in matrixes other than the brain is also possible. In addition to CSF, neurosteroids have often been analysed in plasma [102–105]. Several factors affect neurosteroid levels, such as stress, sex, and health status.

Steroids are commonly extracted from biological matrixes by LLE extraction using diethyl ether or dichloromethane as the organic phase, or SPE extraction on a C-18 phase, or a combination of these [106,107]. As conjugated steroids cannot be analysed by GC-MS, they are commonly hydrolyzed prior to analysis enzymatically by the enzymatic extract from the snail *H. Pomatia*, which contains both glucuronidase and sulfatase activity [108], or by β-glucuronidase derived from *Escherichia coli* [109], chemically [13,110], or directly by derivatization, which causes simultaneous deconjugation [111].

1.3.2 GC-MS

Gas chromatographic analysis of steroids usually requires derivatisation to improve the thermal stability and volatility of the analytes. After hydrolysis of any conjugates, the remaining polar groups are commonly derivatised, mostly by silylation to trimethylsilyl (TMS) conjugates prior to GC-MS analysis [10,112]. Traditional GC-MS methods have utilized ionization in vacuum, such as electron impact (EI) or chemical ionisation (CI). Electron ionization (EI), which is commonly used in the GC-MS analysis of steroids, provides high sensitivity and reproducible spectra for all types of compounds, and it enables the use of EI spectral libraries [101,113–115]. However, EI is an energetic ionization technique, and many compounds such as the relatively labile derivatives of steroids are strongly fragmented and the formation of molecular ions is weak. CI provides less energetic ionization for steroids, and both

positive CI [116,117] and negative CI [98,118,119] have been successfully used in the analysis of steroids by GC-MS.

1.3.3 LC-MS

LC-MS is increasingly favored as it allows the analysis of intact steroids and their conjugates without derivatization or hydrolysis of the conjugates [120]. All the common atmospheric pressure ionization techniques, i.e., ESI [121,122], atmospheric pressure chemical ionization (APCI) [123,124], and atmospheric pressure photoionization (APPI) [125–127], have been applied in the analysis of steroids by LC-MS. Since the ionization efficiency of ESI is relatively low for nonpolar steroids, these are often derivatized, e.g. with hydroxylamine, to achieve sufficient sensitivity. On the other hand, ESI enables the analysis of intact conjugates, such as glucuronides and sulfates [101,128]. APCI, and more particularly APPI, provide better ionization efficiency than ESI for nonpolar steroids, and high sensitivity can be achieved without the need for derivatization. However, APCI and APPI are more energetic ionization processes than ESI, and conjugates are usually cleaved during the ionization [128,129].

Despite the advantages of LC-MS techniques, GC-MS continues to be widely used in the analysis of steroids, despite the need for derivatization and hydrolysis. GC-MS is more robust than LC-MS, and although LC methods are compatible with atmospheric pressure ionisation, the resolving power of GC still exceeds that of LC, giving it an advantage in the separation of steroid isomers, for example. Thus the coupling of GC to API ionization is of high interest.

1.3.4 COUPLING GC TO API MASS SPECTROMETRY

As early as 1973, Horning et al. [130] interfaced GC to MS by using a ^{63}Ni APCI source. Later, APCI with a corona discharge needle [131], APPI [132–134], ESI [135,136], and plasma ionization [137] have been successfully used in the interfacing of GC to MS. Commercially available GC-APCI-MS interfaces are also available nowadays. Our group has developed miniaturized APCI (μAPCI) and APPI (μAPPI) GC-MS interfaces utilizing microfabricated, heated nebulizer microchips and their potential in the analysis of steroids, polycyclic aromatic hydrocarbons, amphetamines, polychlorinated biphenyls, and selective androgen receptor modulators (SARMs) has been demonstrated (Table 2) [138–141]. However, the fabrication of heated nebulizer microchips requires advanced microfabrication technology and clean-room facilities, and therefore we wanted to combine GC to MS via an APPI interface constructed from simple commercially available hardware.

Table 2. *Examples of GC-MS methods using API ionization*

Method	Ionization	Mass spectrometer	Analytes	Matrix	Ref.
GC-MS/MS	µAPPI	QQQ	Anabolic steroids	Urine	[141]
GC-MS/MS	µAPPI	QQQ	PAH:s		[138]
GC-MS/MS	µAPPI/ µAPCI	QQQ/orbitrap	SARM:s	Urine	[140]
GC-MS/MS	ESI	QQQ	MDMA, MDEA, others		[135]
GC-MS	ESI	Q	Volatile organic solvents		[136]
GC-MS/MS	µAPCI	QQQ	Anisole, benzaldehyde + others		[142]
GC-MS	APPI	LIT-orbitrap	Perfume sample		[143]
GC-MS	APCI	TOF	Metabolic fingerprinting	Bacterial culture	[144]
GC-MS	APCI	TOF	Amino acids + others	Human CSF	[145]
GC-MS	MPPI	TOF	Caffeine, nicotine + others		[146]
GCxGC-MS	APCI	TOF	Flame retardants		[147]

µAPPI/µAPCI=APPI or APCI utilizing a nebulizer microhip, Q=single quadrupole, QQQ= triple quadrupole, TOF= time-of-flight mass spectrometer, GCxGC=two-dimensional GC, LIT=linear ion trap

2 AIMS OF THE STUDY

The aim of this work was to develop sensitive and specific mass spectrometric methods for the analysis of neurotransmitters and their glucuronide and sulfate conjugates in microdialysis and cerebrospinal fluid samples. Additionally, a quantitative method for the analysis of neurosteroids in biological samples was developed.

The more detailed aims were:

- to develop and validate a quantitative UPLC-MS/MS methods for the analysis of human brain microdialysates and CSF samples (I)
- to develop an UPLC-MS/MS method for the analysis of intact neurotransmitter glucuronides and sulfates (I)
- to evaluate whether the sulfate conjugates of dopamine (DA-3- and DA-4-S) are able to permeate the blood-brain barrier (IV)
- to develop a sensitive and quantitative GC-MS/MS method for the analysis of neurosteroids by coupling of GC to atmospheric pressure photoionization (II, III)

3 MATERIALS AND METHODS

3.1 CHEMICALS

All chemicals used in this study were analytical or chromatographic grade. The structures of the compounds studied are shown in figures 1 and 2.

3.2 SAMPLES AND PRETREATMENT PROCEDURES

3.2.1 HUMAN BRAIN MICRODIALYSIS SAMPLES

Human brain microdialysis samples (publication I) were obtained from the Neurointensive care unit of the Uppsala University Hospital from two patients with acute brain injuries (subarachnoid hemorrhage). Altogether 172 fractions were collected from a female patient, aged 71 years (patient 1). The sample volume was about 5-10 µL per fraction, collected in a time-resolved mode from day 1-8. The other patient was also female, aged 50 years (patient 2); 132 fractions of similar volume were collected from patient 2 in a time-resolved mode from day 1-6. Both patients had a decreased level of consciousness, were intubated and received artificial ventilation. Intracerebral microdialysis sampling was initiated in conjunction with the insertion of an ICP monitoring device through microdialysis catheters inserted via a bur hole placed 1–2 cm anterior to the coronal suture.

Microdialysis catheters with a membrane length of 10 mm and a 20 kDa nominal molecular weight cut-off polyamide membrane (70 Brain Microdialysis Catheter; M Dialysis AB, Solna, Sweden) were used. The outflow hydrostatic pressure of the perfusion system was set at the zero mid-cranial reference level by taping the collecting vials next to the bandage on the patient's head. Perfusion of the catheters was performed using artificial CSF (Perfusion Fluid CNS, M Dialysis AB), containing NaCl 147 mM, CaCl2 1.7 mM, KCl 2.7 mM, MgCl 0.85 mM, total chloride contents 153.8 mM, osmolarity 305 mOsm/kg), delivered at a rate of 0.3 µL/min by using a microdialysis pump (106 MD Pump, M Dialysis AB). At least 2 hours passed between insertion of the probe and the start of sampling to allow for normalization of changes due to probe insertion. The samples were stored at -70°C until the analysis. The samples were injected as such and the neurotransmitters and their glucuronide and sulfate conjugate contents were measured.

The sampling was approved by the Regional Research Ethics Committee at Uppsala University, and a written informed consent was obtained from the patient or the patient's closest relative, in case the patient was unconscious.

3.2.2 HUMAN CEREBROSPINAL FLUID SAMPLES

Human ventricular cerebrospinal fluid samples (publication I) were obtained from the Department of Neurosurgery at Helsinki University Central Hospital. The samples were obtained by ventriculostomy, and approximately 10 mL of ventricular CSF was obtained from each patient. The patients were being treated in the neurosurgical intensive care unit for obstructive hydrocephalus. The patients were an 82-year old male with a cerebellar infarct (patient 3), a 46-year old female with subarachnoid hemorrhage (patient 4), a 35-year old male with a cerebellar infarct (patient 5), and a 55-year old female with a tumor (patient 6). The samples were taken from CSF waste accumulated during therapeutic CSF drainage (appr. 200 ml/day/patient). Since the samples were taken from CSF waste and were going to be discarded as a part of the clinical routine, no informed consent from the patients or the next of kin was deemed necessary.

A pool of CSF from 200 subjects, all without a neurologic or psychiatric disease, most who underwent lumbar puncture for non-diagnostic reasons and who had normal CSF clinical laboratory values, was obtained from the Neurointensive care unit at the Uppsala University Hospital (publication I). Ages of the patients ranged from 16 to 65 years with a median of 44 years; 50:50 female: male. These samples were collected on ice and cells were removed by centrifugation. Approval for the conduct of this study was obtained from the local Ethics Committee at Uppsala University as well as Göteborg University, Sweden. The participants provided their written informed consent to participate in this study. A written informed consent was obtained from the next of kin, caretaker, or guardian on the behalf of participants that were not able to sign the informed consent themselves. The ethics committees approved of this consent procedure.

All CSF samples were kept at -70 °C until analysis. After thawing, the samples were ultrafiltered by centrifugation (Millipore Amicon Ultrafree-MC, 30 000 NMWL : 12 000 g, 15 min), and the filtrate was injected as such. All CSF samples were analyzed in triplicate.

3.2.3 RAT BRAIN MICRODIALYSIS SAMPLES

Rat brain microdialysis samples (publication IV) were obtained from Wistar rats at 8-12 weeks of age. The rats were housed in groups of four to five per cage and had free access to chow and water. They were maintained under a 12:12 h light/dark cycle with lights on from 06:00 to 18:00 at an ambient temperature of 20-22 °C before the experiments.

The animals were implanted with a guide cannula (BAS MD-2250, Bioanalytical Systems Inc., IN) using a stereotaxic device (Stoelting, Wood Dale, IL) under isoflurane anesthesia (4.5 % during induction for 5 min and then 3.5 % during surgery). The guide cannula was aimed above the rat dorsal striatum (A/P +1.0, L/M 2.7, D/V-6.0) according to the atlas by Paxinos and Watson [148]. The cannula was fastened to the skull with dental cement

(Aqualox, Voco, Germany). A microdialysis probe (BAS MD-2200, 2 mm membrane, Bioanalytical Systems Inc., IN) was inserted into the striatum through the guide cannula on the morning of the experimental day. The protocols were approved by the National Animal Experiment Board of Finland.

The collection of microdialysis samples with 30 min intervals (2.5 μL/min) began 1 h after insertion of the probe. Two baseline samples were collected prior to the injection of a solution of 10 mM DA and 10 mM DA-S (containing the regioisomers DA-3-S and DA-4-S) at a volume of 1 ml/kg body weight (animals 1-3), or 10 mM ^{13}DA-S (containing the regiosisomers ^{13}DA-3-S and ^{13}DA-4-S) at a volume of 1 ml/kg body weight (animals 4-7). After the injections microdialysis samples were collected for 3 hours. The microdialysis samples were stored in a freezer (-70 °C) before analysis with UPLC-MS/MS. The samples were injected as such without sample pretreatment.

3.2.4 HUMAN URINE SAMPLES

Human urine samples (publications II and III) were obtained from four healthy volunteers (three females, one male). Each sample was divided into three aliquots, for the analysis of free, glucuronidated, and sulfated neurosteroids. Free neurosteroids were analyzed by adding 125 mg of $NaHCO_3/K_2CO_3$ (2:1, w/w) to 2.5 mL of urine to adjust the pH to about 8. Subsequently, 4 mL of diethyl ether and 1.5 g of anhydrous sodium sulfate were added, the samples were centrifuged, and the organic layer was separated and evaporated to dryness. Finally, 50 µL of derivatization reagent (MSTFA:NH4I:DTE, 1000:2:4, v/w/w) was added, and the samples were incubated at 60 °C for 15 minutes. The samples were injected into the GC as such.

Glucuronide-conjugated neurosteroids were hydrolyzed by adding 1 mL of 0.8 M sodium phosphate buffer (pH 7) and 50 µL of β-glucuronidase from E. coli. The samples were then incubated at 50 °C for 1.5 hours, cooled to room temperature, and treated similarly to the free fraction samples. Sulfate conjugates were hydrolyzed by adding 50 mg of L-cysteine and 500 µL of 6 M HCl to the urine samples, which were incubated at 100 °C for 30 minutes. Then 275 µL of 10 M NaOH was added and, after cooling to room temperature, the samples were treated in the same way as the free fraction. Methyltestosterone was used as the internal standard in all samples.

3.2.5 SYNTHESIS OF REFERENCE COMPOUNDS

The phase II metabolites of 5-HT, DA, and their phase I metabolites (Table 3), which were used as reference standards in publications I and IV, had been synthesized earlier in our laboratory by methods described in detail elsewhere [24,47,149]. Chemical synthesis of $^{13}C_6$-dopamine-3- and $^{13}C_6$-dopamine-4-sulfates (^{13}DA-3-S and ^{13}DA-4-S) (publication IV) was performed by adding

cold concentrated H_2SO_4 (200 μL) to 20 mg of $^{13}C_6$-DA HCl. The reaction mixture was kept in ice for 20 minutes and then pipetted over 1 mL of frozen water. The pH of the reaction mixture was adjusted to 3 with 5 M NaOH. The sulfates were fractionated, evaporated to dryness under vacuum, lyophilized and reconstituted in Ringer's solution. The synthesis process is described in more detail in publication IV.

3.2.6 UGT EXPERIMENTS

The glucuronidation activity of 19 human UGTs of subfamilies 1A, 2A, and 2B were screened towards 5-HT and HVA (Publication I). All UGTs used had been expressed in our laboratory as described earlier [150–152]. Glucuronidation activities were determined with 2 mM 5-HT or HVA, and 5 mM UDPGA, 50 mM phosphate buffer pH 7.4 and 10 mM $MgCl_2$, and the samples were incubated at 37 °C for 60 min. All UGTs were screened as duplicates, except for 5-HT as a substrate for 2B15, which was analyzed as a single sample and showed no activity. Negative control samples, including all the reaction assay components, with the exception of UDPGA, were also analyzed.

3.3 ANALYTICAL METHODS AND INSTRUMENTATION

3.3.1 LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY

The UPLC used for the analysis of microdialysis and CSF samples was an Aquity UPLC (Waters, Milford, MA). The column used was a pentafluorophenyl column (Thermo Scientific Gold PFP Hypersil, 2.1 x 150 mm, 1.9 μm). Detailed conditions are listed in Table 3.

An Agilent 6410 triple-quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA) equipped with an electrospray ion source was used as the detector in all analyses. Nitrogen (Parker Balston N2-22 nitrogen generator, Parker Hannifin Corporation, Haverhill) was used as the nebulizer (40 psi), curtain (12 L/min, 350 °C), and collision gas. The fragmentor voltages and collision energies were optimized for each compound, and Agilent MassHunter software versions B.04.00 or B.06.00 (quantitative data analysis) and B.03.01 or B.05.00 (qualitative data analysis) were used for data acquisition and processing.

Table 3. *UPLC-MS/MS method for neurotansmitter analysis*

Analyte	SRM transitions	Ionization	UPLC settings
DA	154→137, 119, 91	ESI +	UPLC: Waters Aquity MS: Agilent 6410 Injection volume: 15µl Flowrate: 300µl/min time (min) % org 0-1.5 3 1.5-12 15 12-13.2 65 13.5-25 3
DA-G	330→137, 154,91	ESI +	
DA-S	232→152, 122	ESI -	
HVA	181→137, 122	ESI -	
HVA-G	357→193, 113, 175, 313	ESI -	
HVA-S	261→217, 80	ESI -	
DOPAC	167→123, 95	ESI -	
DOPAC-G	343→299, 123, 113	ESI -	
DOPAC-S	247→203, 123	ESI -	
5-HT	177→160, 115, 132	ESI +	
5-HT-G	353→160, 336, 177	ESI +	
5-HT-S	257→240, 160, 115	ESI +	
5-HIAA	190→146, 144, 116	ESI -	
5-HIAA-G	366→146, 131, 113	ESI -	
5-HIAA-S	270→226, 146, 80	ESI -	
^{13}C-DA-G	336→143, 160, 97	ESI +	Neurotransmitter ^{13}C-metabolites (publication IV)
^{13}C-DA	160→143, 125, 97	ESI +	
^{13}C-DA-S	238→158, 128	ESI -	
^{13}C-DOPAC-G	349→305, 129, 113	ESI -	
^{13}C-DOPAC	173→129, 101	ESI -	
^{13}C-DOPAC-S	253→209, 129	ESI -	
^{13}C-HVA-G	363→193, 113, 175, 319	ESI -	
^{13}C-HVA-S	267→223, 80	ESI -	
^{13}C-HVA	187→143, 128	ESI -	

3.3.2 GAS CHROMATOGRAPHY-MASS SPECTROMETRY

A HP 5890 II gas chromatograph (Hewlett–Packard, Waldbronn, Germany) was used for the chromatographic separation of the neurosteroids both in the GC-APPI-MS and the GC-CPI-MS setups. Two analytical columns, a TR-5MS (length 25 m, i.d. 0.25 mm, 5% phenyl and 95% dimethyl polysiloxane, film thickness 0.25 µm), and a TR-50MS (length 15 m, i.d. 0.25 mm, 50% phenyl and 50% methyl polysiloxane, film thickness 0.25 µm), both from Thermo Fischer, were connected to each other with a press-fit connector (BGB Analytik AG, Boeckten, Switzerland) in the GC-APPI-MS setup; in the GC-CPI-MS setup a BXP5 column (length 15 m, i.d. 0.25 mm, from SGE Europe Ltd., Milton Keynes, UK, was used). The more detailed analysis conditions are listed in Table 4. A methyl-deactivated fused-silica precolumn (1.5 m×0.25 mm i.d.) was connected in front of the analytical column, and a deactivated fused-silica transfer capillary (ca. 1 m×0.15 mm i.d.) after the analytical column, both with

press-fit connectors. The carrier gas was 99.996% pure helium (AGA, Espoo, Finland) with 150 kPa column pressure.

3.3.2.1 GC-APPI-MS interface

The fused-silica transfer capillary connected after the analytical column was fed through a stainless steel (SS) T-piece placed inside the GC oven and then through a SS capillary (i.d. 0.5 mm) acting as transfer line to the MS. The SS transfer line was connected to the SS T-piece and heated to 330 °C with a self-made resistance wire heater. Nitrogen was used as an auxiliary gas at 80 mL/min. Chlorobenzene, used as dopant, was pumped with a syringe pump (PHD 2000; Harvard Apparatus, Holliston, MA) at a flow rate of 3.5 μL/min, vaporized, and mixed with the nitrogen flow. The nitrogen–dopant gas mixture was led through 1/16" o.d. SS tubing into the T-piece, and coaxially through the heated transfer line to the APPI source. The tip of the fused silica transfer capillary from the GC was positioned with a zyx-manipulator (Proxeon Biosystems A/S, Odense, Denmark) ca. 5-10 mm in front of the tip of the MS extended capillary.

The Agilent 6410 triple-quadrupole mass spectrometer was also used as the detector in this study, now utilizing APPI ionization. The MS was equipped with a commercial extended transfer capillary (KR Analytical Ltd., Sandbach, UK). Nitrogen was used as curtain (12 L/min, 350 °C) and collision gas. The fragmentor voltages and collision energies were optimized for each compound. Ionizing 10 eV photons were generated by a krypton rf discharge vacuum ultraviolet (VUV) lamp (PKR 100, Heraeus Noblelight Analytics Ltd., Cambridge, UK). SRM transitions and the GC temperature program are shown in Table 4.

3.3.2.2 GC-CPI-MS

The CPI device consisted of a 1.5 mm i.d. stainless steel (SS) capillary (called CPI capillary) with a 1 mm wide and 15 mm long opening, a flat SS plate with a similar opening hard-soldered on the capillary, a 3 mm thick MgF2 window (Thorlabs Sweden AB, Goteborg, Sweden), a top plate with an 18 mm circular opening, and Teflon rings used as seals to hold the MgF_2 window in place. The capillary (bottom) side of the setup was painted with heat-resistant black paint and heated with IR radiation from a halogen lamp driven by a DC power supply (ISO-TECH IPS2010, RS Components, Northants, U.K.). The injection end of the CPI capillary was heated with a resistance wire heater, also driven by a DC power supply (ISO-TECH IPS603, RS Components). An rf-excited 10 eV (124 nm) krypton discharge vacuum ultraviolet (VUV) lamp (PKR 100, Heraeus Noblelight Analytics Ltd., Cambridge, U.K.) was used to initiate photoionization.

The mass spectrometer in the CPI experiments was an Agilent 6330 ion trap MS (Agilent Technologies, Santa Clara, CA) with a commercial capillary extension (KR Analytical Ltd., Sandbach, U.K.). The CPI setup was connected to the capillary extensionby inserting the extension a few millimeters into the CPI capillary.

Table 4. *GC-MS/MS method for neurosteroid analysis*

Analyte	SRM transitions	Fragmentor (V)	GC settings
DHEA	432→327, 237	180	GC: HP 5890 II
DT	434→405, 127	180	MS: Agilent 6410
T *	432→301, 209	210	Columns: TR-5MS (25m), TR-50MS (15m)
AN	430→234, 209	180	Injector: 250°C
E1	414→309, 155	195	Injection volume: 3 µl, splitless
E2 *	416→285, 129	180	
AP	462→195, 157	180	
MT(istd) *	446→356, 301	180	
IP	462→267, 157	180	Temperature program:
PREG	460→265, 157	180	Isothermal 1 min 190°C
17-OHPREG	584→231, 230	185	12 °C/min 240°C
E3	504→386, 296	180	1 °C/min 255°C
5α-DHP	460→195, 157	180	12 °C/min 330°C
5α-THDOC *	550→243, 230	180	Isothermal 2.4 min 330°C
PROG *	458→353, 157	180	
A	648→305, 233	180	Ionization: APPI
CS	630→435, 243	180	* APPI and CPI
11-DC	544→245, 147	180	
CORT	634→349, 230	180	
HC	632→234, 219	230	

4 RESULTS AND DISCUSSION

This chapter summarizes the results of this work; more detailed descriptions can be found in publications I-IV. The analysis of neurotransmitters was performed with a more traditional UPLC-MS/MS method, although the microdialysis sample matrix and the baseline concentrations of the neurotransmitters required a very sensitive method with good chromatographic separation. In addition to the many compounds included in the analysis, Ringer's solution contains high amounts of inorganic salts, which had to be separated from the analytes chromatographically and prevented from reaching the mass spectrometer, as they cause ion suppression. Concerning neurosteroid analysis, the focus was on developing a new interface between GC and MS, making it possible to combine GC to MS by building an interface for ionization at atmospheric pressure, but as also neurosteroids are present in the brain at low concentrations, the developed method had to be sensitive and selective.

4.1 ANALYSIS OF HUMAN BRAIN MICRODIALYSIS AND HUMAN CSF SAMPLES

4.1.1 UPLC-MS/MS METHOD DEVELOPMENT

An UPLC-MS/MS method was developed for the analysis of DA, 5-HT, and their phase I and phase II metabolites in human brain microdialysis and CSF samples. The use of a pentafluorophenylpropyl column, instead of a commonly used C-18 column, provided good retention and separation of the metabolites from each other, and also from the inorganic salts in the Ringer's solution. The salts were directed to waste using column switching before the elution of the analytes, in order to avoid contamination of the ion source.

The UPLC method provided high separation resolution for the analytes, as well as for the regioisomers, DA-3-sulfate (DA-3-S) and DA-4-sulfate (DA-4-S), in addition to HVA-O-glucuronide (HVA-O-G) and HVA-COO-glucuronide (HVA-COO-G) (Figure 3). Electrospray ionization (ESI) in the positive ion mode provided high ionization efficiency for 5-HT, DA, 5-HT-glucuronide (5-HT-G), 5-HT-sulfate (5-HT-S), and DA-glucuronide (DA-G), whereas the negative ion mode provided better ionization efficiency for DA-3-S and DA-4-S, as well as for DOPAC, HVA, 5-HIAA, and their glucuronide and sulfate conjugates (Table 3) . The positive and negative ion ESI mass spectra showed abundant $[M+H]^+$ and $[M-H]^-$ ions, respectively, which were chosen as the precursor ions. The identification of the neurotransmitters and their metabolites was based on the comparison of the retention times and relative abundances of 2-3 selected reaction monitoring (SRM) transitions (Table 3) of

each analyte between the reference standards diluted in Ringer's solution and the authentic samples. The samples were analyzed in two runs using the same chromatographic gradient but monitoring different SRM transitions in order to maximize sensitivity and selectivity, Run 1 and Run 2 (Figure 3).

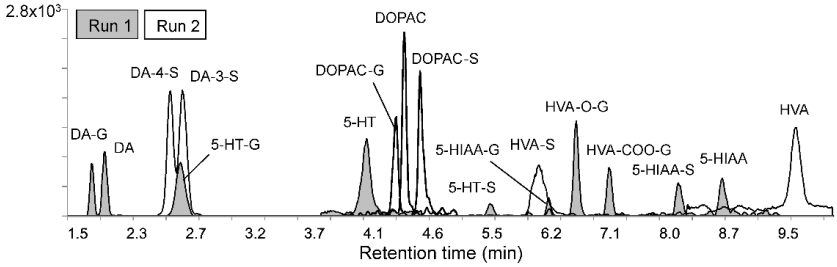

Figure 3 SRM ion chromatograms of the monoamine neurotransmitters and their metabolites.

The UPLC-MS/MS method was validated for specificity, limit of detection (LOD) with a signal-to-noise ratio (S/N) of 3, limit of quantification (LOQ) with a S/N of at least 10 and at most a 20 % deviation from the linearity curve, linearity, and repeatability, using standards diluted in Ringer's solution (Table 5). The LODs for 5-HT, DA, 5-HT-G, 5-HT-S, and DA-G analyzed in the positive ion mode were 0.02-0.40 nM (0.3-6 fmol injected on the column). The LODs for DA-3- and DA-4-S, HVA, DOPAC, 5-HIAA, and their glucuronides and sulfates in the negative ion mode varied between 0.3 and 25 nM (4.5-375 fmol injected on the column). The LODs for DA and 5-HT and their metabolites were at the same level as described earlier [24,45,47,83,87]. With the UPLC column (2.1 x 150 mm, 1.9 µm) an injection volume of 15 µL was used in order to achieve sufficient sensitivity levels.

Concerning the microdialysis samples, the recoveries of the neurotransmitters and their phase I and II metabolites across the microdialysis membrane were not determined, but the typical in vivo recovery for small molecules (glucose, lactate, pyruvate, glutamate) in microdialysis has been reported to be 65-70% [153]. The concentration ratios between the analytes are expected to be similar to those in brain fluid, since the permeability of the analytes through the microdialysis membrane is not selective.

Table 5. *Validation results for the neurotransmitters and their metabolites*

Compound	Linearity range nM	r^2	LOD S/N=3 nM	LOQ S/N≥10 nM	Accuracy %	Repeatability 10 nM, n=6 rsd %	Repeatability 100 nM, n=6 rsd %
DA	0.5-25	0.997	0.2	0.5	90-120	6.0	nm
DA-G	0.5-50	0.998	0.1	0.5	90-110	4.3	nm
DA-3-S	1-50	0.997	0.3	1	91-116	5.1	0.6
	50-1000	0.998			93-115		
DA-4-S	1-1000	0.998	0.3	1	91-112	5.7	1.5
5-HT	1-25	0.996	0.2	1	89-112	1.8	nm
5-HT-G	0.25-25	0.997	0.02	0.25	96-103	2.5	nm
5-HT-S	1-100	0.999	0.4	1	94-107	2.6	nm
DOPAC	50-1000	0.998	10	50	95-110	nm	4.7
DOPAC-G	25-1000	0.996	2.5	25	93-113	nm	2.0
DOPAC-S	25-1000	0.998	4.3	25	95-110	nm	nm
HVA	100-1000	0.999	25	100	98-102	nm	7.0
	250-5000	0.999			95-120		
HVA-O-G	5-100	0.999	1.0	5	92-111	4.9	2.9
HVA-S	5-500	0.999	1.4	5	95-118	nm	nm
5-HIAA	25-1000	0.998	6.4	25	91-106	nm	4.0
	250-3500	0.999			95-103		
5-HIAA-G	5-500	0.998	1.0	5	86-116	7.2	3.2
5-HIAA-S	10-1000	0.999	5.0	10	96-113	nm	10.0

nm= not measured

4.1.2 NEUROTRANSMITTERS AND THEIR METABOLITES IN HUMAN BRAIN MICRODIALYSIS AND CSF SAMPLES

The human brain microdialysis samples from two patients (patients 1 and 2) were analyzed as such. 5-HT was identified at low concentrations (< 1 nM) in the microdialysis samples from patient 2, but not from patient 1. DA was not detected in any of the samples. The most abundant metabolite of DA was HVA, but HVA-O-glucuronide was also identified in the samples obtained from patient 1. DA-3-S was clearly identified in both patients, but DA-4-S was not found in any of the samples. The most abundant metabolites of 5-HT were 5-HIAA and 5-HIAA-S, but low concentrations of 5-HT-G were also identified in the samples from patient 1 (Figure 4).

Low concentrations of 5-HT (1.2 nM or less) were detected in three of the ventricular CSF samples similar to those found in the brain microdialysis samples. In comparison, DA was not detected in any of the samples. The most abundant metabolite of DA was HVA, the concentrations of which were about one order of magnitude higher in the ventricular samples than in the lumbar sample. DA-3-S was detected in all the samples but DA-4-S only in one sample. The most abundant metabolites of 5-HT were 5-HIAA and 5-HIAA-S, which corresponded to those found in the brain microdialysis samples. 5-HT-S was

detected at a low concentration only in the lumbar CSF sample, but 5-HT-G that was detected in the microdialysis samples was not detected in any of the CSF samples.

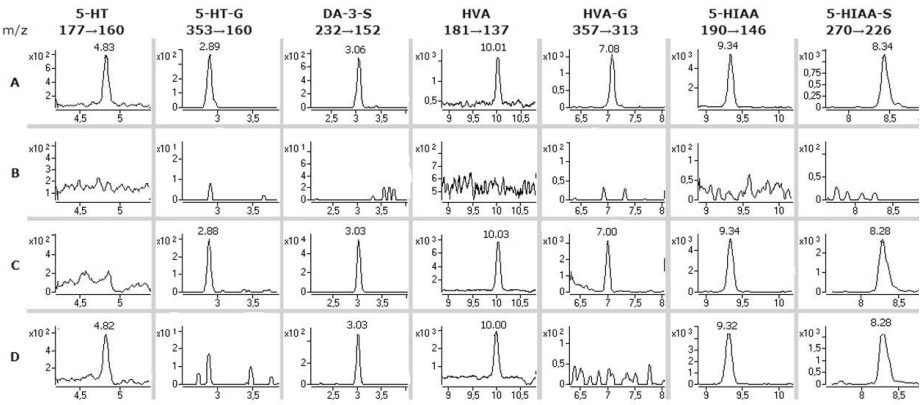

Figure 4 SRM ion chromatograms of A: a standard sample (1 nM 5-HT and 5-HT-G, 5 nM DA-3-S and HVA-O-G, 100 nM HVA, 5-HIAA and 5-HIAA-S); B: a blank sample (Ringer's solution); C: a microdialysis sample from patient 1; D: a microdialysis sample from patient 2.

The findings show that the main phase I metabolites of DA and 5-HT were HVA and 5-HIAA, respectively. This is consistent with earlier studies of their concentrations in human brain tissue [93,94,154–156] and human CSF samples [65,93,95,157]. Earlier studies reported that DOPAC was detected in human brain tissue samples [154–156,158], microdialysis samples [58,92], and CSF samples [65,93,95], but none was detected in this study. This may be due to insufficient sensitivity of the method or to fast degradation of DA and DOPAC during sample storage, as reported earlier by several groups [24,159,160].

The intact phase II metabolites, 5-HIAA-S, DA-3-S, DA-4-S, 5-HT-S, 5-HT-G, and HVA-O-G were detected in human brain or CSF for the first time in this work (Table 6). There are earlier reports of HVA-G, DOPAC-G, 5-HIAA-G, and DOPAC-S in tissue samples of the human brain [161], and DA-G, 5-HT-G, 5-HT- S [162], and DA- S [95,157,160,162–164] have been found in human CSF. All of these metabolites were detected after a hydrolysis step, however, which makes their determinations less reliable than our direct method of detecting intact conjugates. In addition 5-HIAA-S, which has not been detected in the human brain or CSF in earlier studies, was now found at relatively high concentrations. The concentrations of 5-HIAA-S were around 3-5 times higher than those of free 5-HIAA in the human microdialysis and lumbar CSF samples, and about half the concentration of free 5-HIAA in the ventricular CSF samples.

DA-3-S was clearly detected in all the samples and DA-4-S in only one sample, which indicates that the DA-3-S regioisomer predominates in the

human brain. These results are in accordance with earlier observations that DA-3-S predominates in human plasma at concentrations of about ten-fold higher than those of the regioisomer DA-4-S [165]. It has been shown that the isoenzyme SULT1A3, which is also found in the human brain[21], is regioselective and strongly favors the formation of DA-3-S over DA-4-S [149]. Sulfonation of 5-HT was less favored than sulfonation of DA, as 5-HT-S was only detected in the lumbar CSF sample at a low concentration.

The glucuronides 5-HT-G and HVA-O-G were detected for the first time in human brain samples. However, the glucuronides were only found in the microdialysis samples of patient 1 at low concentrations, and no glucuronides were detected in the CSF samples. The amount of HVA-O-G was less than 1% of the amount of free HVA. This result may be considered to be in line with the reported 5-HT glucuronidation of UGT1A6 [166] and the barely detectable expression of this enzyme in the brain [16,18].

Table 6. *Findings in the human brain microdialysis and cerebrospinal fluid samples.*

	Microdialysis samples				Cerebrospinal fluid samples				
	Patient 1		Patient 2		Ventricular				Lumbar
Compound	Sample 1	Sample 2	Sample 1	Sample 2	Patient 3	Patient 4	Patient 5	Patient 6	Pooled
5-HT	-	-	0.52[a]	0.54[a]	0.84[a]	-	0.70[a]	1.2	-
5-HT-S	-	-	-	-	-	-	-	-	0.63[a]
5-HT-G	0.45	0.80	-	-	-	-	-	-	-
5-HIAA	90	110	90	100	1400	340	220	540	35
5-HIAA-S	450	550	520	380	370	170	96	390	86
5-HIAA-G	-	-	-	-	-	-	-	-	-
DA	-	-	-	-	-	-	-	-	-
DA-3-S	330	630	3.5	2.5	10	18	14	9.4	12
DA-4-S	-	-	-	-	-	-	2.0	-	-
DA-G	-	-	-	-	-	-	-	-	-
HVA	1100	2000	280	150	4200	600	2200	2200	120
HVA-S	-	-	-	-	-	-	-	-	-
HVA-O-G	15	12	-	-	-	-	-	-	-
DOPAC	-	-	-	-	-	-	-	-	-
DOPAC-S	-	-	-	-	-	-	-	-	-
DOPAC-G	-	-	-	-	-	-	-	-	-

The microdialysis samples (patients 1 and 2) were analyzed as singles. Each of the CSF samples (four ventricular CSF samples (patients 1–4), one lumbar CSF sample pooled from several patients, were analyzed in triplicate (concentrations shown are the mean concentrations). – not detected, [a]<LOQ, concentration estimated by extrapolation of the linearity curve.

4.1.3 UGT SCREENING EXPERIMENTS FOR 5-HT AND HVA

Since this was the first study to show the presence of intact glucuronide conjugates of 5-HT and HVA in a human brain sample, the glucuronidation activity of 19 human UGTs of subfamilies 1A, 2A, and 2B were screened for 5-

HT and HVA in order to evaluate the relevance of the finding. 5-HT glucuronidation has been previously well documented [16,166,167], but the conjugation of HVA by recombinant UGTs is elusive. In this study, serotonin was shown to be conjugated mainly by UGT1A6, but also by the UGTs 1A7-1A10 in minor amounts. This was almost in agreement with previous results, though low levels of UGT2B7 activity have also been observed for 5-HT [151].

HVA can be glucuronidated both at the phenolic hydroxyl and the carboxylic acid group sites [24]. This screening experiment revealed that UGT1A10 is the most active human UGT in the formation of HVA-O-glucuronide, whereas UGT2A1 is the most active for the conjugation of HVA at the –COOH-group (UGT screening experiment described in more detail in publication I). The glucuronidation results showed that glucuronidation is not a major biotransformation pathway for 5-HT and HVA in the human brain. In general, the results suggest that conjugation with sulfonate is the major phase II metabolism pathway in the human brain.

4.2 PERMEATION OF DOPAMINE SULFATE THROUGH THE BLOOD-BRAIN BARRIER

Even though several sulfonate conjugates of neurotransmitters were detected in the human brain, the origin of these was not studied. It is not known whether the conjugates were locally formed in the brain, or whether they can cross the BBB. Therefore the BBB permeation of DA and its sulfonate conjugates was studied by peripheral (subcutaneous) injections of DA, DA-3- and DA-4-S, as well as $^{13}C_6$-isotopically labeled DA-3-S (^{13}DA-3-S) and DA-4-S (^{13}DA-4-S) in rats, while the concentrations of these compounds in the brain were simultaneously monitored by microdialysis. The fate of the injected compounds, and their possible effects on the concentrations of DA, 5-HT and their metabolites, were studied by analyzing the rat brain microdialysis samples using ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS).

4.2.1 SYNTHESIS OF ^{13}C-LABELED DOPAMINE SULFATE

The structures of the synthesized ^{13}DA-3- and ^{13}DA-4-S were verified by UPLC-MS and UPLC-MS/MS using negative ion mode. The ion chromatograms showed two peaks, the MS spectra of which showed abundant $[M-H]^-$ ions at m/z 238 indicating the correct molecular weights of the synthetized ^{13}DA-3-S and ^{13}DA-4-S. The product ion spectra of the $[M-H]^-$ showed abundant product ions $[M-H-SO_3]^-$ (m/z 158) and minor fragments $[M-H-CH_2NH_2]^-$ (m/z 128) and $[SO_3]^-$ (m/z 80) (Figure 5). Based on UV detection, the mixture of the ^{13}DA-S regioisomers contained 53% of ^{13}DA-4-S and 47% of ^{13}DA-3-S. The sulfation sites were verified by NMR. The spectra of the DA-S

regioisomers are in accordance with earlier reported NMR data [149] confirming sulfonation to the hydroxyl groups attached to carbons 3 and 4.

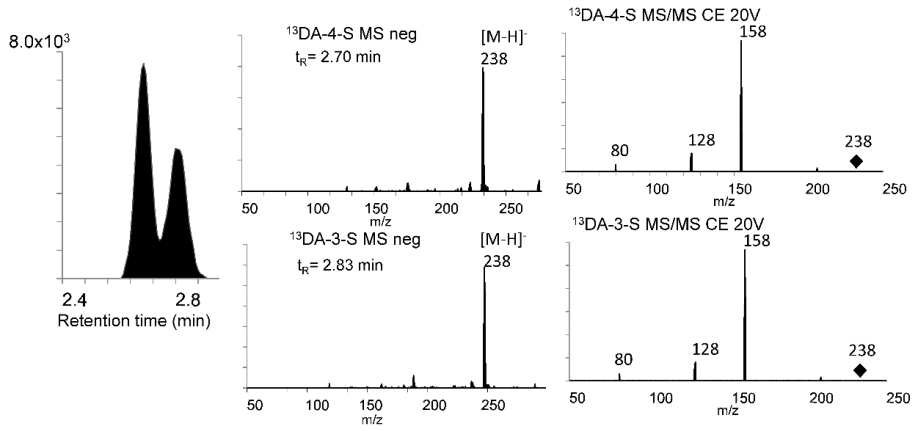

Figure 5 Extracted ion chromatogram, MS and MS/MS spectra (negative ion mode) of the synthesized ^{13}DA-S.

4.2.2 BBB PERMEATION EXPERIMENT

A mixture containing DA-S (DA-3-S and DA-4-S, 1:1) 2.3 mg/kg and DA 1.5 mg/kg was injected subcutaneously (s.c) and the concentrations in rat brain were followed by microdialysis. Microdialysis samples were collected at 30 minutes intervals for 3 hours after administration (n=3, Rats 1-3). By also injecting intact DA, the function of the BBB was controlled, as DA does not permeate the BBB. In all three rats the concentrations of DA-3-S and DA-4-S were below the limit of detection before the injection, but increased clearly 30 min after the injection (Figure 6). To confirm the results, the experiment was repeated with a mixture of labelled ^{13}DA-S regioisomers (2.4 mg/kg) (n=4, Rats 4-7). Again, in all four rats, the concentrations of both ^{13}DA-3-S and ^{13}DA-4-S increased clearly 30 min after the s.c. injection. In contrast to DA-S, the concentrations of intact DA did not change significantly after the injection, measuring at the same levels as reported earlier in rat striatal microdialysis samples (0.7-8 nM) [24,47,49]. These results clearly show that DA-S permeates the intact BBB.

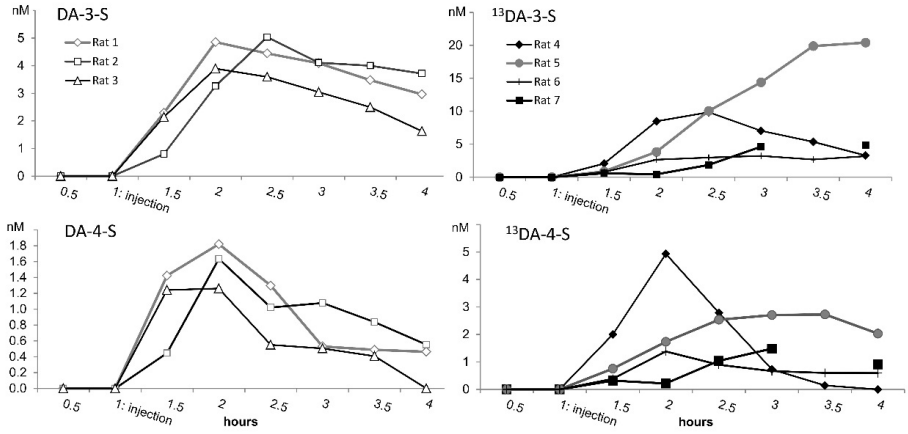

Figure 6 Concentrations of non-labeled and $^{13}C_6$-labelled DA-3-S and DA-4-S in rat brain microdialysates after s.c. injections.

4.2.3 EFFECT OF THE INJECTED DA-S ON THE CONCENTRATIONS OF OTHER NEUROTRANSMITTERS IN THE BRAIN

As the pharmacological properties of DA-S are are unknown, despite some reported properties such as convulsions after intraventricular injections [168,169], the concentration profiles of the other naturally occurring neurotransmitters, non-labelled DA, 5-HT, and their metabolites (Table 3), were studied in microdialysis samples collected before, during, and after the injection of $^{13}C_6$-labelled DA-S. The basal concentrations of the naturally occurring analytes measured before the injections were in accordance with earlier published values for rat brain microdialysis samples collected from freely moving rats [24,47,50,88], even though this experiment was performed with anesthetized rats.

The concentrations of the naturally occurring non-labelled metabolites of dopamine (DA-G, DOPAC, DOPAC-S, HVA and HVA-S) increased after the injections of $^{13}C_6$-labelled DA-S, while no clear trend in the levels of DA could be observed. 5-HT and its metabolites followed a similar trend: while the 5-HT levels decreased in all animals during the experiment, the concentrations of its metabolites (5-HT-G, 5-HIAA and 5-HIAA-S) increased during the experiment (Figure 7). The injected ^{13}DA-S may affect the metabolism process of naturally occurring dopamine and/or other neurotransmitters, but anesthesia itself might also have an impact on the concentration changes. The influence of volatile anesthetics on neurotransmitter levels have been reported earlier: isoflurane has been shown to lower 5-HT levels in rats and mice compared to levels during wakefulness [170,171]. Isoflurane anesthesia has also been shown to increase DA levels at high doses (3% isoflurane), while the levels of DOPAC and HVA increased at all isoflurane concentrations studied [172,173]. Similar effects have been reported for halothane and sevoflurane [174,175]. Based on the literature it seems likely that the fluctuations in

neurotransmitter levels are due to anesthesia. Moreover, the isoflurane concentrations had to be slightly adjusted during the experiments, which might have additionally influenced the neurotransmitter levels. However, this does not rule out that the injected DA-S might also have contributed to these level changes.

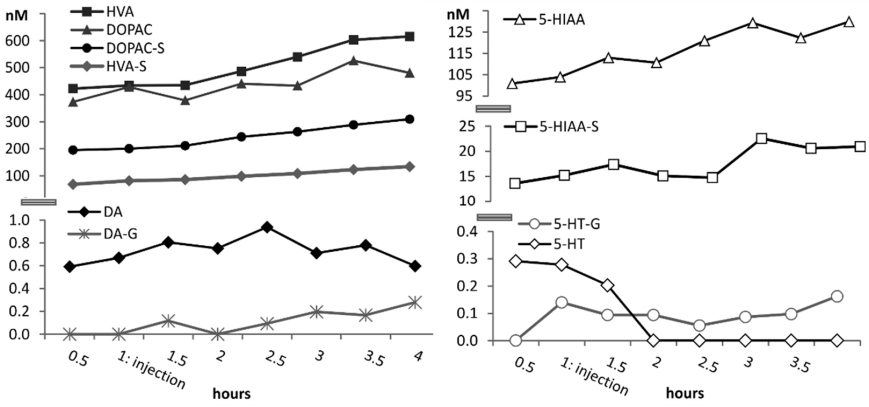

Figure 7 Concentrations of the neurotransmitters (Left: dopamine metabolites, right: serotonin metabolites) during the test in rat brain microdialysates in one of the test animals.

4.3 DEVELOPMENT OF A GAS CHROMATOGRAPHIC – TANDEM MASS SPECTROMETRIC ANALYSIS METHOD FOR NEUROSTEROIDS USING ATMOSPHERIC PRESSURE PHOTOIONIZATION

Miniaturized APPI (μAPPI) GC-MS interfaces utilizing microfabricated, heated nebulizer microchips have been successfully used in the analysis of steroids [141,176]. In this work we combined GC to MS via a new APPI interface constructed from simple commercially available hardware. The feasibility of the method was demonstrated in the analysis of trimethylsilylated (TMS) neurosteroids in human urine samples. The interface was further modified to a capillary photoionization interface (CPI), in which ionization takes place inside a heated transfer capillary between the GC and the MS inlet, thus maximizing ion transmission.

4.3.1 CHROMATOGRAPHIC SEPARATION

High chromatographic separation power is essential in the GC-MS/MS analysis of steroids to separate the numerous isobaric and isomeric forms. The

separation of co-eluting isobaric and isomeric steroids is often impossible by MS/MS, since fragmentation behavior may be similar, and specific product ions are not necessarily formed. Furthermore, M+1 and M+2 isotopes and fragments of trimethylsilyl-derivatized steroids may disturb the analysis. In our GC-MS/MS method, the use of two different GC columns (TR-5MS and TR-50MS) connected in tandem provided high chromatographic resolution and sufficient analytical specificity (Figure 8).

The hydroxyl and keto groups of the steroids were converted into trimethylsilyl (TMS) ethers and enol ethers following previously described procedures [177,178]. The chromatographic separation of the compounds had to be optimized carefully, since several of the neurosteroids eluted as two chromatographically separated isomeric peaks. This was due to an acetyl group at position 17 (PROG, PREG, 5α-DHP, IP, AP and 17-OH-PREG) or a hydroxyacetone group in the same position (5α-THDOC and A), the ketone group of which may form an enol to either side of the double bond (Z- and E-isomers) during derivatization, with resultant elution of two chromatographic peaks [113]. Aldosterone (A) additionally has an aldehyde group at position 13, which is not derivatized owing to the formation of the hemiacetal isomer [179].

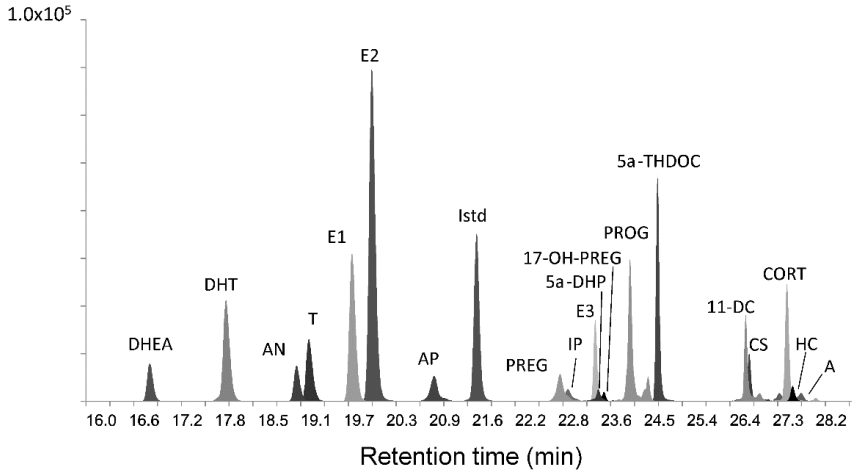

Figure 8 SRM chromatograms of the neurosteroids.

4.3.2 POSITIONING OF THE GC TRANSFER LINE AND KRYPTON DISCHARGE LAMP

As the interface between GC and MS was self-built and a the ionization region was completely open, the GC transfer line and the VUV lamp had to be arranged carefully in order to maximize ion transmission into the MS. The positions of the tip of the heated transfer line from the GC, which was positioned with a zyx-manipulator, and the krypton discharge vacuum UV

(VUV) lamp in relation to the extended capillary of the MS inlet were crucial in achieving maximum sensitivity. The optimal position for the end of the GC transfer line was 5 to 10 mm from the tip of the extended capillary. Stability of the signal was best when the flow from the GC was directed toward the tip of the extended capillary at an angle of about 45° (Figure 9). The lamp was positioned as close as possible (about 2 mm) to the sample plume generated at the end of the transfer line. A longer distance between the UV lamp and plume allowed absorption of the photons by air, decreasing the intensity of radiation at the plume and the ionization efficiency. The flow rates of the dopant and the auxiliary gas (used for dopant vaporization) were optimized, and optimal sensitivity and stability were achieved with dopant and auxiliary gas flow rates of 3.5 µL/min and 80 mL/min, respectively.

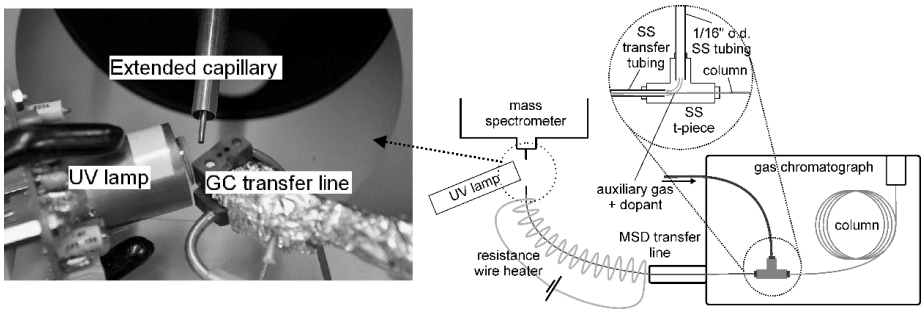

Figure 9 Schematic (right) of the GC-APPI-MS/MS connection setup and picture (left) showing the MS inlet. Reprinted from [180] with permission from Elsevier.

4.3.3 DOPANTS

The effects of several dopants (chlorobenzene, toluene and anisole) on ionization of the TMS ($Si(CH_3)_3$) derivatives of the steroids were compared. With all dopants and for most TMS-steroids, the APPI-MS spectra showed abundant molecular ions ($M^{\bullet+}$), formed via charge exchange reaction, with minimal fragmentation. When chlorobenzene was used as dopant, the TMS-steroids produced no or only minor amounts of protonated molecules ($[M+H]^+$), as shown by the same or only slightly higher proportions of the [M+1] peaks relative to the theoretical isotopic abundances. The use of toluene or anisole, however, resulted in the formation of $[M+H]^+$, as seen in the higher relative abundances of [M+1] peaks than with chlorobenzene (Figure 10). The ionization energy of chlorobenzene (9.07 eV) is higher than that of toluene (8.82 eV) or anisole (8.20 eV)[181], which favors charge exchange reactions and the formation of $M^{\bullet+}$ ions. Many unknown factors, such as the formation of cluster ions and gas phase reactions with the dopant, could, however, affect the reactant ion composition and thus the ionization process. Because chlorobenzene provided good ionization efficiency and a more uniform ionization process than toluene or anisole, it was selected as the dopant for further experiments.

Under similar experimental conditions, nonderivatized steroids have been demonstrated to produce abundant $[M+H]^+$ rather than $M^{\bullet+}$ ions [141]. The probable explanation for the different results is that increase in molecular size through TMS derivatization of the hydroxyl and keto groups tends to decrease the ionization energy of the steroids [182,183]. The trimethylsilyl groups also lower the proton affinity of the analytes by binding to the possible protonation sites. Moreover, the TMS group causes steric hindrance for the proton transfer to the ether oxygen. Thus, the TMS derivatives of the studied steroids are more likely ionized by charge exchange than by proton transfer reaction.

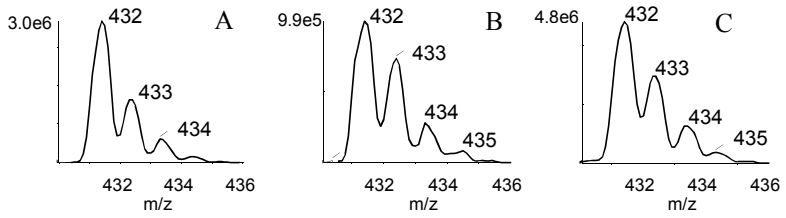

Figure 10 Spectral peak distribution of testosterone with A) chlorobenzene, B) toluene and C) anisole as dopant. Reprinted from [180], with permission from Elsevier.

4.3.4 MASS SPECTROMETRY

The MS spectra of almost all the TMS-derivatized steroids measured with chlorobenzene as dopant showed an $M^{\bullet+}$ ion as the main peak. Exceptionally, corticosteroids, which have a hydroxyacetone side chain and a hydroxyl group at position 17 (11-DC, CS and HC), showed a $[M\text{-}TMS\text{-}H_2O]^+$ fragment ion as the main peak and, additionally, a minor fragment ion $[M\text{-}Si(CH_3)_2]^+$. This indicates that when a hydroxyacetone side chain is present at position 17, TMS-derivatives of the hydroxyl group at position 17 are less stable than the other TMS-derivatized groups. Those steroids with a hydroxyacetone side chain but no hydroxyl group at position 17 show a single peak of $(M^{\bullet+})$ in their spectra. For most of the compounds, the fragmentation was low, and most of the fragments, such as $[M\text{-}TMS]^+$, $[M\text{-}Si(CH_3)_2]^+$, and $[M–CH_3]^+$, were formed through dissociation of the TMS group.

$M^{\bullet+}$ was selected as the precursor ion for all analytes except 11-DC, CS, and HC, for which $[M\text{-}TMS\text{-}H_2O]^{\bullet+}$ was selected, and A, for which $[M\text{-}TMS]^{\bullet+}$ was selected. In addition to abundant nonselective ions formed by the loss of a methyl group or the loss of TMS and water, the MS/MS spectra also showed numerous and abundant specific fragment ions formed by the dissociation of the ring structure of the steroid. The fragmentation follows the odd-electron fragmentation pathway, and therefore the product ion spectra of the TMS-derivatized steroids are closely similar to the corresponding EI spectra (Figure 11), and EI libraries could thus be utilized in the identification of unknown compounds.

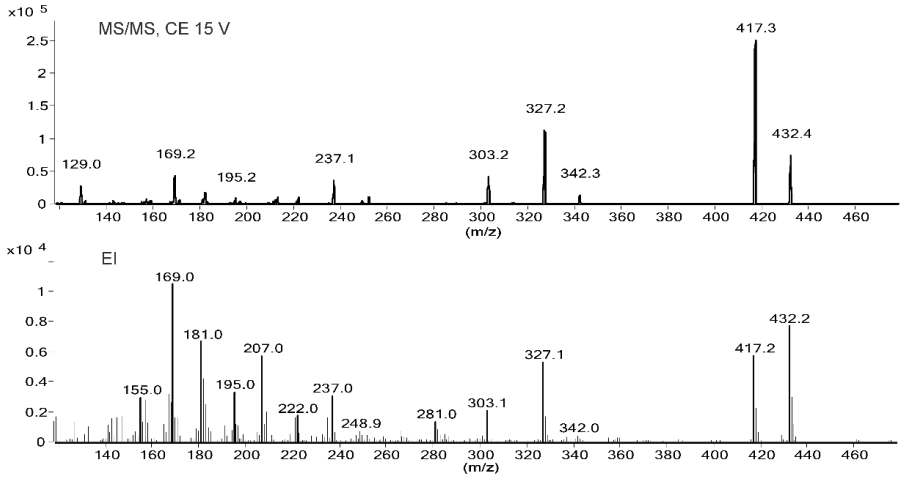

Figure 11 GC-APPI-MS/MS and GC-EI spectra of DHEA. The EI spectra was measured with an Agilent 6890N GC connected to an Agilent 5975B MSD mass spectrometer using an electron energy of 70 eV.

4.3.5 VALIDATION OF THE METHOD AND ANALYSIS OF URINE SAMPLES

Spiked artificial urine samples were used for method validation. The steroids were extracted from the urine samples with diethyl ether as described previously [184,185]. The validated parameters were linearity, limits of detection and quantification (LOD and LOQ), repeatability, and liquid–liquid extraction (LLE) recovery. For linearity measurements, the artificial urine samples were spiked with the analytes at concentrations from 0.25 pg/mL to 5000 ng/mL. The LLE recovery was measured by comparing two artificial urine samples, one spiked with standards at 10 ng/mL before LLE and one spiked at the same level after LLE (triplicate samples). The extraction recoveries varied between 43 and 87%, which is adequate, as the repeatability of the method was acceptable with relative standard deviations better than 22%.

The LODs were 0.1-0.2 ng/mL for progestagens (PREG, PROG, 17-OH-PREG), 0.02-0.1 ng/mL for estrogens (E1, E2, E3), 0.02-0.1 ng/mL for androgens (DHEA, AN, T, DHT), 0.75-1.0 ng/mL for glucocorticoids (11-DC, HC, CS), and 0.2-10 ng/mL for mineralocorticoids (A, CORT). LODs reported in the literature for GC-EI/MS are in the range of 0.06-3 ng/mL for progestagens [105,113,186], 0.08-0.16 ng/mL for estrogens [105], 0.06-2.5 ng/mL for androgens [105,113,114,186], and 0.07 ng/mL for corticosteroids [113]. The sensitivity of our method is thus well comparable with sensitivities presented in the literature. The within-day repeatability of the method was studied at concentration levels of 20 and 50 ng/mL, by spiking analytes into artificial

urine (six replicates). The relative standard deviations (% rsd) were below 15%, indicating good repeatability. The results are shown in Table 7.

The performance of the GC-APPI-MS/MS-method was verified by analyzing human urine samples. Each sample was divided into three aliquots: one used for the analysis of the free fraction of steroids, one analyzed after enzymatic hydrolysis of glucuronides with β-glucuronidase, and one analyzed after acid hydrolysis of sulfates. The steroids were detected mostly as conjugates (Table 8). The results agree well with an earlier study [187], in which 3α-hydroxysteroids (such as AP and 5α-THDOC) were show to be conjugated mainly with glucuronic acid; 3β-hydroxysteroids (such as PREG and DHEA) conjugated with sulfate; and 17β-hydroxysteroids (T, E2, E3 and DHT) could be conjugated with both glucuronic acid and sulfate. In addition to PREG and DHEA, which were excreted as sulfate conjugates, AN was mostly, and CORT completely, excreted in this form. The corticosteroids CS and HC, which are slightly less lipophilic than androstanes and pregnanes, were the only steroids detected in an unconjugated form in all samples. This finding is consistent with earlier results [188].

Table 7. *Validation results for the neurosteroids*

Compound	Linearity ng/ml	r^2	LOD S/N=3-5 ng/mL	LOQ S/N ≥10 ng/mL	Repeatability, n=6		LLE extraction recovery %
					20ng/mL rsd %	50ng/mL rsd %	
11-DC	1-100	0.992	0.75	1.0	8.9	4.1	87
17-OHPREG	1-100	0.997	0.1	1.0	8.4	6.5	61
5α-DHP	10-500	0.996	5.0	10.0	5.0	22.4	53
5α-THDOC	0.5-10	0.999	0.01	0.5	12.7	10.0	63
A	100-2500	0.990	10.0	100.0	1.1	1.5	85
AP	2-100	0.997	0.2	2.0	7.3	3.6	50
AN	0.2-10	0.999	0.02	0.2	2.6	2.9	67
CORT	2-50	0.994	0.2	2.0	2.3	1.1	61
CS	5-250	0.994	1.0	5.0	3.1	2.1	57
DHEA	2-100	0.996	0.2	0.75	9.8	11.6	69
DHT	0.5-20	0.998	0.1	0.2	13.1	3.7	70
E2	0.2-10	0.993	0.1	0.2	13.7	4.8	66
E3	0.5-10	0.998	0.02	0.5	15.0	6.2	70
E1	0.5-5	0.994	0.05	0.5	14.2	6.8	58
HC	2-100	0.995	0.75	2.0	2.0	4.8	69
IP	1-100	0.994	0.75	1.0	7.6	6.3	43
PREG	1-50	0.998	0.2	1.0	2.9	3.3	53
PROG	0.5-10	0.994	0.1	0.5	5.8	6.0	55
T	0.2-10	0.997	0.1	0.2	11.9	3.0	70
T	5-250	0.997					

Table 8. *Concentrations (ng/mL) of the steroids in urine samples from three females and one male. The samples were analyzed in duplicate and average values are presented.*

Free fraction	**11-DC**	**17-OH-PREG**	**5α-DHP**	**5α-THDOC**	**A**	**AP**	**AN**	**CORT**	**CS**	**DHEA**	**DHT**	**E2**	**E3**	**E1**	**HC**	**IP**	**PREG**	**PROG**	**T**
Female 1	-	-	-	-	-	-	-	-	16.7	-	-	-	-	-	10.1	-	2.7	-	-
Female 2	-	-	-	-	-	-	-	-	43.1	-	-	-	-	-	33.5	-	-	-	-
Female 3	-	-	-	-	-	-	-	-	46.9	-	-	-	-	-	26.1	-	-	-	-
Male 1	-	-	-	-	-	-	1.4	-	74.9	-	-	-	-	-	86.6	-	-	-	0.3
Glucuronide fraction	**11-DC**	**17-OH-PREG**	**5α-DHP**	**5α-THDOC**	**A**	**AP**	**AN**	**CORT**	**CS**	**DHEA**	**DHT**	**E2**	**E3**	**E1**	**HC**	**IP**	**PREG**	**PROG**	**T**
Female 1	-	-	-	1.0	-	20.2	0.6	-	23.5	6.4	1.4	0.2	1.0	1.5	15.7	-	2.3	-	-
Female 2	-	-	-	1.4	-	24.6	-	-	85.5	20.6	0.9	0.4	2.0	2.2	51.0	-	-	-	2.5
Female 3	-	-	-	1.3	-	29.2	1.0	-	70.1	7.9	0.6	0.5	2.4	3.2	31.0	-	1.0	-	1.6
Male 1	-	2.7	-	3.7	-	33.2	2.5	-	134.8	41.3	3.6	1.2	5.0	5.8	73.7	-	-	-	38.7
Sulfate fraction	**11-DC**	**17-OH-PREG**	**5α-DHP**	**5α-THDOC**	**A**	**AP**	**AN**	**CORT**	**CS**	**DHEA**	**DHT**	**E2**	**E3**	**E1**	**HC**	**IP**	**PREG**	**PROG**	**T**
Female 1	-	-*	-	-	-	38.4	5.3	5.3	-	12.6	0.5	*0.04**	-*	*0.6**	-	-	*2.7**	-	1.1
Female 2	-	-*	-	-	-	64.3	19.1	5.3	21.6	212.1	0.2	*0.17**	*0.7**	*0.8**	22.4	-	*11.7**	1.9	3.7
Female 3	-	-*	-	-	-	44.5	7.1	4.5	15.3	192.7	0.2	*0.1**	-*	*1.2**	-	-	*11.3**	-	1.3
Male 1	-	-*	-	-	-	122.7	22.2	7.0	37.4	426.7	1.0	*0.3**	*1.9**	*1.5**	24.1	-	*47.5**	2.1	30.7

* these compounds were significantly decomposed under the acid hydrolysis conditions and the results may not reflect actual levels in the samples

4.3.6 CAPILLARY PHOTOIONIZATION

The developed APPI interface for GC-MS showed good potential for the analysis of steroids, but the interface was still a self-made prototype with a completely open ionization region. Ionization efficiency and ion transmission are the key factors affecting the sensitivity of mass spectrometric analysis utilizing atmospheric pressure ionization (API). When analytes are ionized at atmospheric pressure, most of the ions are lost when transferred to the vacuum of the mass spectrometer [189,190], and as the ionization region in the GC-APPI-MS/MS setup was completely open, a more shielded interface was constructed. Earlier photoionization for generating ions inside a transfer capillary between the atmosphere and the vacuum of the MS has been performed using a miniature spark discharge VUV lamp embedded in a transfer capillary with a windowless aperture (cAPPI) [191].

A high sensitivity ionization method, in which ionization takes place inside a capillary, called capillary photoionization (CPI), was constructed. In the GC-CPI-MS setup, the tip of the SS tubing from the GC was inserted a few millimeters into the CPI capillary. The CPI capillary was connected to the extended capillary of the MS by inserting the extended capillary a few millimeters into the CPI capillary (Figure 12). The sample was introduced (injected manually, or from the GC) into the transfer capillary and vaporized by heat. VUV radiation entered the capillary through the VUV-transparent MgF_2 –window, and ionized the analytes by photoionization. With this CPI-setup, non-volatile and liquid samples can also be ionized.

The sensitivity of CPI-MS was first tested by direct injections. Samples of 2 µL were injected manually, with a 10 µL syringe, about 5 mm inside the heated capillary. Injection speed was about 1 µL/s. In MS/MS mode the LOD for testosterone was at low fmol level.

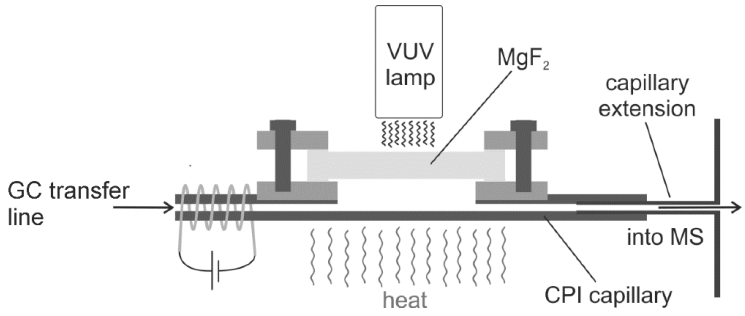

Figure 12 Schematic of the capillary photoionization device connecting GC to an API mass spectrometer. Adapted from [192], with permission from Elsevier.

The performance of GC–CPI-MS/MS was further tested with TMS-derivatized steroids (T, PREG, PROG, E2 and THDOC). As in the GC-APPI-

MS setup, with chlorobenzene as dopant, the TMS-steroids produced intense molecular ions ($M^{+\cdot}$) via charge exchange reaction, with no formation of protonated molecules and minimal fragmentation. No peak broadening or tailing was caused by the CPI interface, due to the high temperature of the setup.

Artificial urine samples spiked with steroids (T, PROG, PREG, E2, and THDOC) were used for the validation of the GC-CPI-MS method. The sample pretreatment was similar to the GC-APPI-MS/MS method. The validated parameters were linearity, limits of detection (LOD), and injection repeatability. The application was verified by analyzing human urine samples from two healthy males. The LODs (with a S/N of 3) for the steroids spiked in artificial urine were between 2 and 6 pg/mL, being lower or equal to those presented for GC-EI-MS/MS [105,113]. Repeatability of injection with GC-CPI-MS/MS was good: RSDs of the peak areas were 7% or less at a concentration of 5 ng/mL. The linearity of the method was measured in the range of 10 pg/mL to 100 ng/mL, and the coefficient of determination (r^2) was higher than 0.993, indicating good linearity.

As the CPI-device is heated, and can also handle liquid samples and nonvolatile compounds, it can be used both for direct liquid injection and for connecting LC to MS. CPI also enables GC instruments to be used with API mass spectrometers intended for LC-MS. The initial results indicated high sensitivity, thanks to the high iontransmission efficiency. The results from the analysis of selected steroids in urine samples show that the method has good potential for being used in applications of analyzing less polar compounds such as steroids in biological samples. Further experiments with GC, LC, and other types of mass spectrometers are needed to demonstrate the full potential of CPI.

5 SUMMARY AND CONCLUSIONS

The microdialyis sampling and the developed UPLC-MS/MS-analysis method require minimal sample pretreatment, and provide sensitive and selective analysis of intact phase II metabolites of neurotransmitters in the human brain. In this study, intact phase II metabolites of 5-HT and DA were analyzed in human brain samples without hydrolysis of the conjugates, and several intact glucuronides and sulfates were unambiguously identified and quantified in human brain microdialysis and CSF samples for the first time. 5-HIAA-S was also determined for the first time in human brain. As the direct method provides analysis of regioisomers of conjugates, we were able to show that DA-3-S predominates over DA-4-S in the human brain. The results show that sulfonation is a more important phase II metabolism pathway than glucuronidation in the human brain. Further studies, including samples from a larger group of patients, are still required to study interindividual differences in the amounts of conjugated neurotransmitters versus the concentrations of the parent compounds.

The role and the origin of the conjugated neurotransmitter in the brain remains unknown. It is also unknown whether the conjugates possess neurotoxic or neuroprotective properties, if they are pharmacologically active or not, and additional experiments are needed to study the pharmacological impact of conjugated and especially sulfonated neurotransmitters on different brain functions. DA-S is assumed to be biologically less active than DA, possibly providing a source for free DA in brain through enzymatic hydrolysis. Therefore, the study of the permeation of DA-S through the human BBB and the fate of DA-S in human brain is of great interest.

The peripheral injections of ^{13}C-labelled DA-S followed by analysis of brain microdialysis samples by UPLC-MS/MS provides a reliable method to study the permeation of DA-S through the BBB. The study clearly shows that DA-S, but not DA, is able to permeate the BBB of rats. The result is interesting, since DA-S is more polar than DA, and the passive permeation of DA-S through the BBB is not obvious. Therefore, it can be concluded that DA-S most likely permeates the BBB by binding to an active transporter-protein located in the BBB. However, it is unknown which transporter protein is responsible for transporting DA-S through the BBB into the brain, which will be a focus of future studies.

Even though the results show that DA-S crosses the BBB, no information on the fate of the permeated DA-S in the brain was acquired. The results showed that DA-S is not hydrolyzed to DA or transformed into any common metabolites of DA with concentrations above the limits of detection of this method. It is therefore possible that DA-S is metabolized by an unknown mechanism, or is transported out of the brain by active transporters. However, these results obtained from experiments with rats cannot be directly applied

to humans. DA-S has been found in human brain microdialysis samples previously [25], but not in rat brain microdialysis samples, indicating that the concentration of DA-S in human brain is significantly higher than in rat brain.

Steroids have recently been analyzed by methods combining gas chromatography (GC) to atmospheric pressure photoionization utilizing heated nebulizer microchips (μAPPI). The interface constructed in this work for combining GC to mass spectrometry (MS) using dopant-assisted atmospheric pressure photoionization (APPI) utilized only commercially available hardware. The developed GC-APPI-MS/MS method provided high chromatographic resolution and efficient ionization of neurosteroids, which formed mainly abundant radical cations (M•+) with minimal fragmentation when chlorobenzene was used as the dopant. The method showed good specificity and sensitivity and is therefore well suited for the analysis of neurosteroids in biological samples. The APPI interface provides an easy means to couple GC to MS, allowing the high chromatographic resolution capacity of gas chromatography to be exploited with any mass spectrometer equipped with an atmospheric pressure ionization interface. The radical cations of the steroids formed by APPI ionization showed fragmentation patterns in their MS/MS spectra similar to the patterns seen in the corresponding EI spectra. Use of EI libraries could thus be possible, enabling the identification of a wide range of unknown compounds.

The GC-APPI-MS interface was nevertheless a self-made prototype, and the interface was further developed in order to maximize ion transmission and thus increase the sensitivity of the method. The presented prototype capillary photoionization device, CPI, showed good potential as a simple and effective ion source for the analysis of both polar and nonpolar compounds. Compared to the GC-APPI-MS/MS method, which employed a completely open ion source, ion transmission is higher with the CPI device, where the ionization region is shielded. Further experiments with GC, LC, and other types of mass spectrometers are needed to demonstrate the full potential of CPI.

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